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Dr. Hideyo Noguchi

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EXPERIMENTAL INTRADERMAL PNEUMOCOCCUS INFECTION IN RABBITS.

By KENNETH GOODNER.

(From the Department of Bacteriology and Immunology of Harvard University Medical School, Boston.)

PLATES 1 AND 2.

The work to be described in this paper is the outgrowth of an observation (1) made in this laboratory a few years ago to the effect that the intradermal inoculation of rabbits with Type I pneumococci produces a characteristic and gradually extending lesion which, representing a sort of "dermal pneumonia," permits direct observation of changes, progressive or healing, under various experimental conditions.

Preliminary experiments showed that this lesion and the various occurrences as nearly resemble the sequences in human lobar pneumonia as do those of any other experimental method (except possibly experimental pneumonia in monkeys). Furthermore, the advantages of a visible lesion and the reliability in its production have led us to believe the technic to be a valuable one.

The production in animals of lesions comparable to lobar pneumonia has been attempted by many investigators. Wadsworth (2) produced typical lobar pneumonia by introducing pneumococci into the respiratory passages of partially immunized rabbits. Many others have employed this technic and modifications of it but such methods are not altogether reliable. Stillman and Branch (3) have produced inhalation pneumonias in mice and rabbits, but these pneumonias are not lobar and the results are not regular. Bull and McKee (4) have produced pneumonias in rabbits by intranasal inoculation. Lobar pneumonia in monkeys, such as that studied by Blake and Cecil (5), has some of the same disadvantages as the rabbit pneumonia and the expense of animals precludes their routine use.

The status of the pneumonia problem has not been materially changed since it was reviewed (1) in 1925. Human pneumococcus

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lobar pneumonia is regarded as a symptom-complex arising from a local infection, complicated in many cases by the entrance of bacteria into the blood stream. Symptoms described clinically as those of toxemia are produced, although the exact cause of this condition is not known and no convincing demonstration of a pneumococcus toxin has been given. The therapeutic use of antisera as now prepared, even in concentrated form, has not proven satisfactory unless employed early in the course of the disease.

We have undertaken to resurvey the subject of pneumococcic infection, using the intradermal rabbit lesion as a basis for experimentation. In this paper we purpose to describe the lesion in detail and give a preliminary report on certain experiments bearing on immunity and serum therapy.

Experimental Methods.

Pneumococcus Cultures.—The Type I pneumococcus used in these experiments was grown in beef heart hormone broth (pH 7.5) to which small amounts of sterile defibrinated rabbit blood had been added. The virulence of this strain for mice was such that 0.000,000,01 to 0.000,000,001 cc. of an 18 hour culture, given intraperitoneally, sufficed to kill within 96 hours. This virulence was usually checked for each new experiment, the tests being made at the time of infecting the rabbits.

Rabbits.—Healthy, light colored female rabbits of 1300 to 2000 gm. were selected. The entire abdominal area of each animal was shaved, care being taken not to injure the skin in any way. The size, sex, or breed of the rabbit is unimportant but it must be of healthy stock. Because of the large amount of technical work involved, the largest convenient number of rabbits for a single experiment is six.

Infection.—After taking the necessary normal readings, the rabbit is given an intradermal inoculation of 0.2 cc. of the desired dilution of 18 hour broth culture. The site of infection is well up on the side of the shaved area, 6–7 cm. lateral to the rabbit's ventral midline.

Observations.—At regular intervals the appearance of the focal skin lesion is described, particularly as to its extent, the color of the various parts, the amount of swelling and the location of the excessively swollen areas, the consistency of the exudate, the amount of wrinkling and contraction of the skin, and the changes in these features from time to time.

Blood Cultures.—Using an accurately graduated tuberculin syringe a definite amount of blood is withdrawn from the rabbit's marginal ear vein. This blood and dilutions of it are plated immediately in cooled hormone agar. Small quantities of fresh, sterile, defibrinated horse blood are added in the case of dilutions. Plate counts obtained in this fashion represent approximately the number of

aggregations of pneumococci per cc. of circulating blood and give a rather accurate comparison of the septic processes at various times in different individuals. If carefully carried out, an almost indefinite number of such blood samples may be taken from the same animal.

Blood Samples Other Than for Cultures.—Since it is important to leave the marginal ear veins intact for obtaining blood for cultures it is necessary to obtain blood samples for other purposes from the smaller vessels of the ear. An area is carefully shaved and cleaned, and a small amount of xylol applied to the tip of the ear. When sufficient dilatation has been brought about, one of the smaller vessels is severed with a sharp razor, gross tissue injury being avoided. The blood flowing from the wound is collected and the serum used for the determination of content of protective substances, agglutinins, precipitins, etc.

Lesion Cultures.—The presence of pneumococci in the primary lesion is determined by culturing fluid aspirated from it. A fine gauge needle is inserted at a point previously treated with alcohol and a small amount of fluid is aspirated into a syringe and transferred to a blood agar plate. Positive results are regarded as more significant than negative for late in the disease, when the animal has lost considerable weight, it is often difficult to obtain fluid from the lesion.

The Focal Lesion Arising after Intradermal Infection.

The lesion usually makes its first appearance at 8 to 12 hours as an inflamed, swollen area 1 cm. in diameter. It progresses ventrally until at 24 hours it occupies a strip 2–3 cm. wide extending from the point of inoculation to, and across, the midline. The area at the midline is especially swollen by the accumulation of serous or serofibrinous exudate. Any further progression is along the ventral midline and the entire midabdominal area may become involved. Fig. 1 shows a drawing of a lesion at 30 hours. In the following paragraphs a detailed description is given.

The time of appearance of the initial lesion is dependent upon the number of organisms injected. With the intradermal inoculation of about 0.001 cc. of an 18 hour broth culture, the injection bleb completely disappears within the first half hour. In 8–10 hours the blood vessels on the infected side show congestion, then in the immediate vicinity of the inoculation the congested vessels lose their sharp outlines and the skin over a 1–2 cm. area takes on a highly inflamed color and becomes swollen. As this development continues the edema is most prominent on the ventral border. The lesion then spreads ventrally, the heightened color being usually preceded by the accumulation of fluid. The amount of swelling and the actual or apparent movement of the serous exudate give the impression that a viscous, unorganized fluid is seeping or flowing ventrally from the force of gravity. At 14–16 hours the edematous area usually has extended to

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the midline. More serous material accumulates in the subepidermal tissues until the entire area is puffy. At 24 hours the lesion usually has a width of not over 3 cm. (as measured along the midline) but in length it extends from the point of inoculation ventrally to the midline and passes this by 2–3 cm. Within the next 24 hours the lesion usually extends along the midline in both directions, although the zone of greatest swelling is still located at the midline ventral to the point of inoculation.

In untreated animals the color of the lesion varies from a pale to a moderately bright orange red. In treated animals the lesion becomes uniformly red after treatment and then the color fades completely.

Depending upon local trauma and upon factors affecting the cutaneous circulation, parts of the surface of the lesion may become necrotic. This necrosis is preceded by petechial hemorrhages, and the area then takes on a greenish hue. Seepage from such areas tends to deplete the total amount of fluid and in such cases secondary infection may occur.

TABLE I.

Distribution of Deaths by Days in a Series of Fifty-Six Untreated Cases.

	Day of death						Re-covered
	2nd	3rd	4th	5th	6th	7th	
No. of rabbits.....................	12	16	6	7	4	3	8
Percentage distribution............	21.4	28.7	10.7	12.5	7.1	5.3	14.3

Within 30–40 hours, even in fatal infections, the skin contracts and folds, large with serofibrinous exudate, develop. The mechanism of this process of contraction probably represents the involvement of the underlying muscles. In one typical case the shaved area had an original length of 13 cm. At 24 hours after infection this was 8 cm., while by 48 hours it was only 5 cm. Owing to the shortening of the abdominal surface the rabbit usually assumes an abnormal posture, with the hind legs drawn up closely under the body.

There is no change in the local lesion before or at death, but with recovery a process of resolution is instituted which is characterized by epidermal desquamation and fading of color. In the course of several days the lesion then disappears completely, and except in the case of previous necrosis no scar remains.

The Disease Other Than in the Focus.

The development of the focal lesion is accompanied by an abrupt rise in temperature and this temperature persists at a high level until

death or recovery. Organisms enter the blood stream within 12 to 24 hours.

The majority of untreated animals die with severe pneumococcus bacteriemia, the distribution of deaths by days being shown in Table I. At autopsy virulent organisms are found in all parts of the body. Save for the skin lesion and a slight enlargement of the spleen there is no significant pathology.

About 14 per cent of untreated cases recover spontaneously on the 5th, 6th, or 7th days. This recovery is preceded by the disappearance of organisms except from the focal lesion and is associated with an abrupt fall in temperature, changes in circulating cellular elements, the appearance in the blood stream of "protective substances," and with changes in the local lesion.

The relationship of these various occurrences to each other is described below.

The Bacteriemia.

In most instances, shortly after the appearance of the local lesion, organisms may be detected in small number in the circulating blood. This number tends to increase so that at 24 hours the aggregations of pneumococci per cc. of circulating blood in the average animal is about 50. In exceptional cases the number is so high as to grossly distort any arithmetical average of the entire series. The number of circulating organisms tends to increase gradually; this rate is not in strict geometrical progression such as would be expected if the bacteria were multiplying in the blood stream. In the typical case the number increases up to a certain point (near 100,000 per cc.) that appears to constitute a lethal level. The time necessary for arriving at this level may be as short as 30 hours but it is more often 60 to 70 hours after infection. In some animals there occur fluctuations in the bacteriemia such that at times no organisms may be isolated. These individuals are apt to ultimately recover. If the lethal level has not been reached in the characteristic period, recovery is associated with or preceded by the disappearance of the organisms from the blood stream.

Charts showing the development of the bacteriemia will be found below.

The Temperature Reaction.

The temperature of the normal rabbit is variable and easily influenced by environment. In a series of 120 rabbits we find temperatures distributed over a range from 101.9° to 103.5°F., with an average of 102.8°F. The temperature of an acutely diseased rabbit undergoes less fluctuation due to environment and is of some significance when correlated with other data. Temperatures over 104.0°F. are regarded as definitely above normal levels.

At 5 to 12 hours after intradermal pneumococcus infection an animal's temperature begins to rise slowly and then abruptly goes up to a high level, usually over 105.5°F. The time occupied in this abrupt rise is ordinarily not over 4 hours. The third phrase is again one of slowly increasing temperature, a maximum being reached in 18 to 24 hours. This maximum ranges from 105.7° to 107.5°F. Then in spite of handling, the temperature remains at a high, though not stationary, level until death or the beginning of the recovery phase. In the case of death the temperature usually remains high until a few minutes before actual death.

In the cases of rabbits which recover, the temperature persists at a high level for 5 to 7 days and then without warning drops to normal. This fall in temperature is rapid and is associated with other signs of recovery, in these respects not unlike what is spoken of as crisis in human pneumonia, another observation which persuades us of the close analogy of our rabbit disease with that of human pneumonia. As will be seen in a later section, again as in human disease, such a "crisis" is definitely correlated to the appearance of protective substances in the blood.

Text-fig. 1 shows charts of characteristic temperature curves in untreated cases.

It has long been believed that the temperature in lobar pneumonia is not related to the septicemia, but it seemed desirable to investigate this point in the experimental condition of the rabbit. An examination of records of a series of over fifty untreated cases shows that the temperature onset may occur (1) at the time when the organisms are first found in the blood stream, and more often this is the case; (2) it may occur as long as 36 hours before the beginning of the bacteriemia
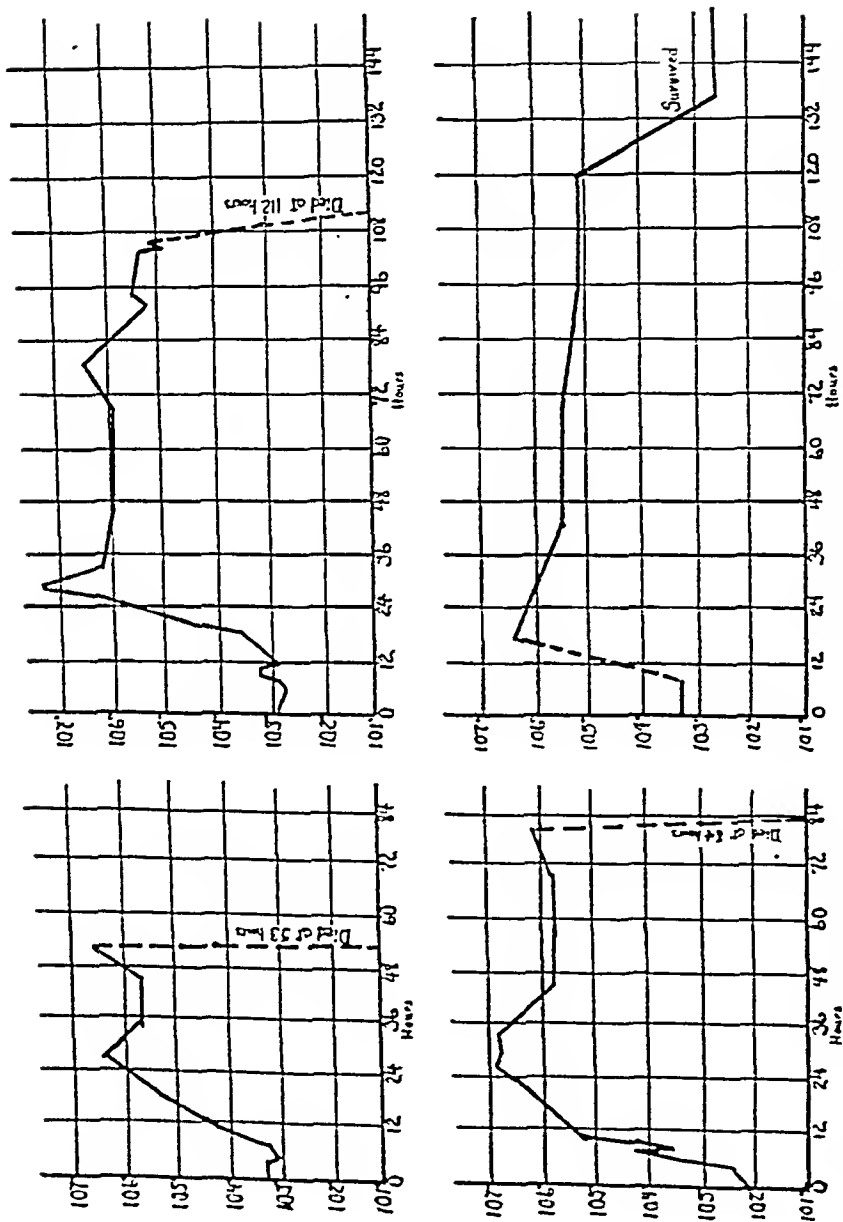
Text-Fig. 1. Characteristic temperature curves in various rabbits, including one instance of survival without treatment. Infection in each case at 0 hour.
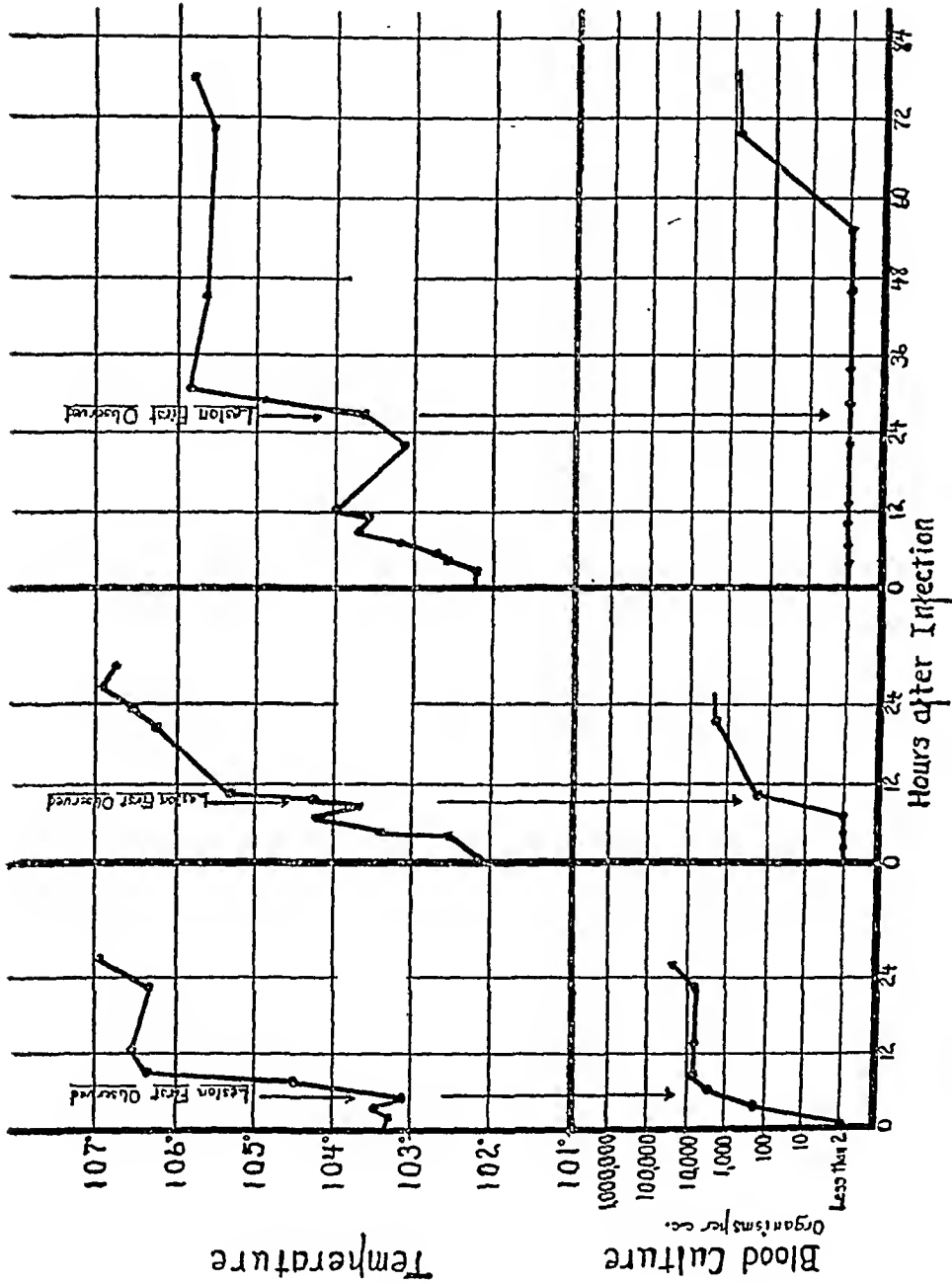
TEXT-FIG. 2. Temperature and bacteriemia determinations on three rabbits. These curves show the independence of the beginning of the bacteriemia and the onset of temperature These three cases reading from left to right represent maximal, average, and minimal intradermal dosage of pneumococci.

(as with minimal dosage); or (3) it may be delayed until several hours after the onset of septicemia (as with maximal dosage). Charts of examples of such findings are given in Text-fig. 2. The temperature onset is therefore independent of the bacteriemia.

From correlations of the appearance of the local lesion with the temperature it is apparent that the temperature rise always occurs exactly at the time that the local lesion is first observed.

The stimulus which brings about the continued febrile reaction is not easily determined. During this phase there are always living organisms in the local lesion, though not necessarily in the blood stream. The level of the temperature during this period is not proportional to the severity of the bacteriemia, nor is it proportional to the intensity of local inflammation, if the latter may be judged by color and edema. During this phase of high temperature the local lesion may undergo shrinkage or necrosis and it may become somewhat solid, but it never shows the cardinal signs associated with recovery: the appearance of surface desquamation and loss of inflammatory color.

The sudden recovery, as marked by the fall in temperature, is not accompanied by the immediate disappearance of virulent organisms from the lesion, but in spite of this the lesion does show the above mentioned recessive signs.

In partially immunized rabbits, the detailed description of which will be found in a later paper, it is possible to give an intradermal injection of pneumococci and obtain a local inflammation. In this case of partial immunity there is little or no increase in temperature.

It seems therefore that the systemic effects indicated by temperature are not correlated with the presence of living organisms either in the blood stream or lesion but with the absorption from the focus, where living organisms are present, of something which either is no longer formed in the recovering or partially immune animal or which being formed is neutralized. We are led from this to believe that in the local lesion there is elaborated some toxic factor which in turn is responsible in large measure for the intensity of the general reaction.

The White Cell Count.

The white count on the rabbit is generally regarded as subject to great fluctuation. The average count in one series of fifty-four
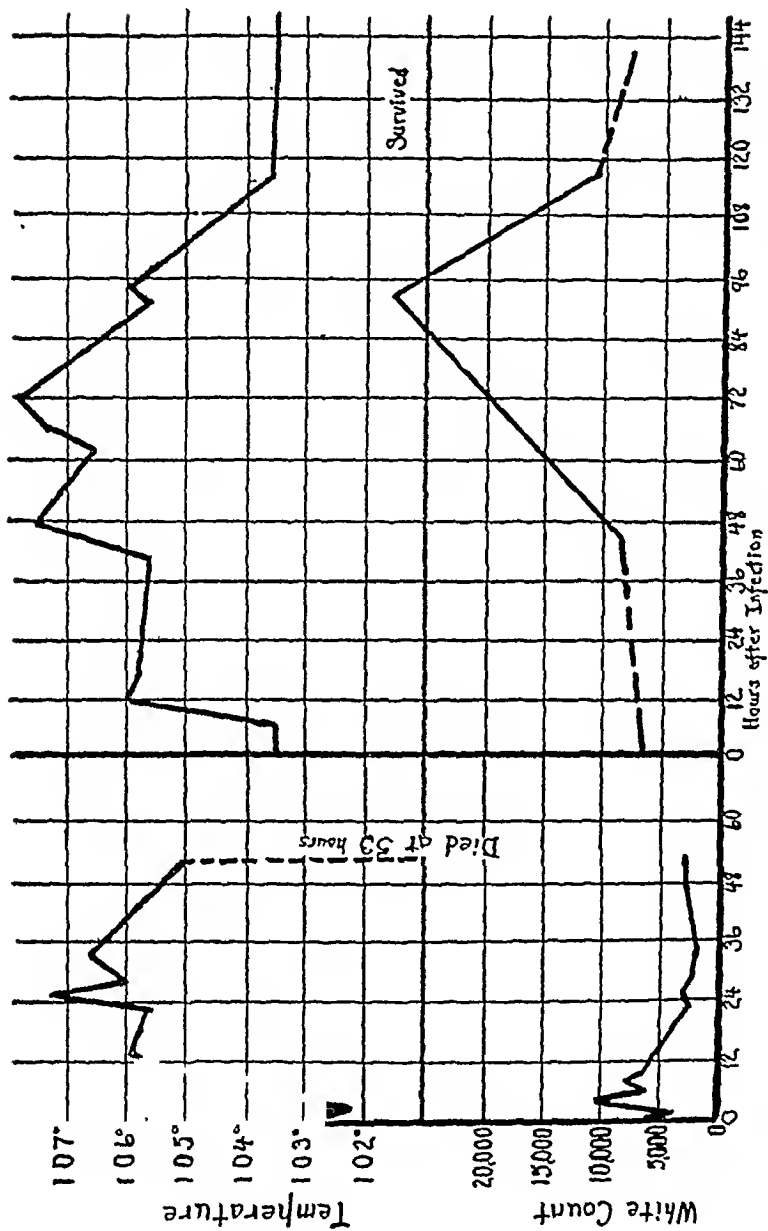
Text-Fig. 3. Temperature and white count curves in two typical cases, one of which survived.

rabbits was 7280, but repeated counts on individual rabbits undergoing handling showed that it was difficult to establish a relative normal for any individual.

The first symptoms following intracutaneous infection are accompanied by a definite leucopenia, the decrease being accomplished largely by the disappearance of polymorphonuclear cells from the blood stream. In animals which recover, this leucopenia persists for 2 or 3 days and is followed by a definite leucocytosis, the number of white cells becoming normal again after the animal's recovery. In the case of animals which die during the course of the disease, the white count usually remains below normal throughout. The white count thus constitutes a definite prognostic sign, though it is by no means infallible. In Text-fig. 3 are shown examples of counts on each type of animal.

In many cases the number of white cells is roughly inversely proportional to the bacteriemia. With organisms circulating in the blood stream the white count is usually low but when the blood is freed of pneumococci the white cell count rises. There is some evidence to indicate that these inverse changes in white count do not accompany changes in the status of bacteriemia but occur only after such a change.

These findings are consistent with the favorable prognostic significance attributed in lobar pneumonia both to the absence of septicemia and to the high white count.

Lesion Cultures.

Cultures of fluids aspirated from the focal lesion in normal rabbits show large numbers of pneumococci during the active stages of the infection. Technical difficulties and late consolidation of the lesion sometimes prevent successful aspirations. At the time of recovery, as marked by various signs, there is a decrease in the number of organisms in the lesion, but virulent organisms may sometimes be isolated as long as a week and certainly for 1 or 2 days after crisis, which again is analogous to the presence of pneumococci in the lungs after crisis as demonstrated by Thomas and Parker (6).

Weight Loss.

One-fifth to one-fourth of the total body weight of the infected rabbit may be lost during the period of high temperature. A definite loss of at least 10 per cent has been an invariable finding. Some of this is undoubtedly due to the failure of the animal to take food but the emaciation initiated during the disease usually persists for several weeks after recovery, in spite of special diet and care.

Development of Protective Substances and Agglutinins.

The spontaneous recovery of untreated typical cases is associated with the appearance in the blood serum of protective substances. By this term we refer to that property of serum by which mice are passively protected against pneumococcic infection, provided that the serum is administered just prior to or at the time of infection. In such instances of critical recovery we have found that the precritical serum possesses no protective property but that serum which is immediately postcritical is protective against at least 0.1 cc. of highly virulent culture. This matter will be reported at greater length in a later paper and evidence will be presented to show that this point (immediately postcritical) may be the zone of greatest passive protective value of the serum.

Agglutinins and precipitins have never been detected in less than 2 days after recovery in the untreated animal. The agglutinins usually attain their highest titer at 12 to 18 days after infective inoculation (5 to 15 days after recovery).

The serum of convalescent rabbits is an effective therapeutic agent in the treatment of other cases. This will be reported on in detail in a later paper.

Pathological Examination.

At postmortem examination the skin at the point of greatest involvement is found to have a thickness varying from 12 to 18 mm., as contrasted to a normal thickness of 2 to 3 mm. On sectioning such a lesion one observes a subepidermal jelly-like mass from which straw colored fluid oozes. The skin at the point of original inoculation is usually slightly if at all thickened.

There is no constant gross pathology besides the skin lesion. In many cases there is a slight enlargement of the spleen but other organs are essentially negative. No perforations between lesion and peritoneum have been observed. It is possible to isolate virulent pneumococci from heart's blood, the serous cavities, and almost all tissues.

Microscopically, sections of the skin lesion show that the epidermis is not involved but that the corium is enormously swollen with serofibrinous material containing numerous cells. There is marked congestion of blood vessels and lymphatics. The subcutaneous connective tissues are usually swollen and here as well as in the muscle layers there is some cellular infiltration. In some cases there is a considerable atrophy of bundles of muscle fibers. Although fluid aspirated from the lesion during life reveals large numbers of diplococci, it is somewhat difficult to demonstrate these in sections. However it is certain that in the untreated lesion the organisms are extracellular. Fig. 2 shows a very low magnification of a cross-section of a pneumococcic skin. Fig. 3 represents a highly magnified detail from such a section. The character of the phlegmon is shown and in one portion there is an injected lymphatic.

The lungs are frequently congested but show no signs of pneumonitis. Virulent pneumococci may be isolated from the meninges post mortem but there is no meningitis.

The Susceptibility of the Rabbit to Minimal Intradermal Infections.

It is generally recognized that the normal rabbit has no resistance against the pneumococcus but opinions differ as to quantitative infectivity: as to whether the necessary infective dose is a function of the weight of the animal, or of the number of virulent organisms in the culture employed.

We have found that the minimal quantity of culture necessary to infect mice intraperitoneally represents also the minimal quantity for intradermal infection of rabbits, that is to say, if a culture has a virulence for mice such that 0.000,000,1 cc. causes infection and death, the same amount constitutes the minimal intradermal dose for rabbits. This result is independent of the weight of the animal—that is, the necessary dosage is not the number of organisms per kilo of body weight but rather of organisms per individual rabbit. With smaller doses the onset of symptoms is delayed to such an extent that it is evident that the number of organisms must multiply in vivo to a certain level before an actual lesion is brought about. The disease resulting from a small dose, though delayed in first appearance, is in no way different from that caused by a larger infecting dose.

When undiluted virulent culture is injected intradermally there occurs as in other cases a definite latent period and then, perhaps 2 hours before the temperature rise and the actual appearance of the lesion, it is possible to isolate organisms from the blood stream. It

might be argued that the effective level was exceeded at the time of infection but that this did not hasten the termination of the latent period necessary for signs of local inflammation.
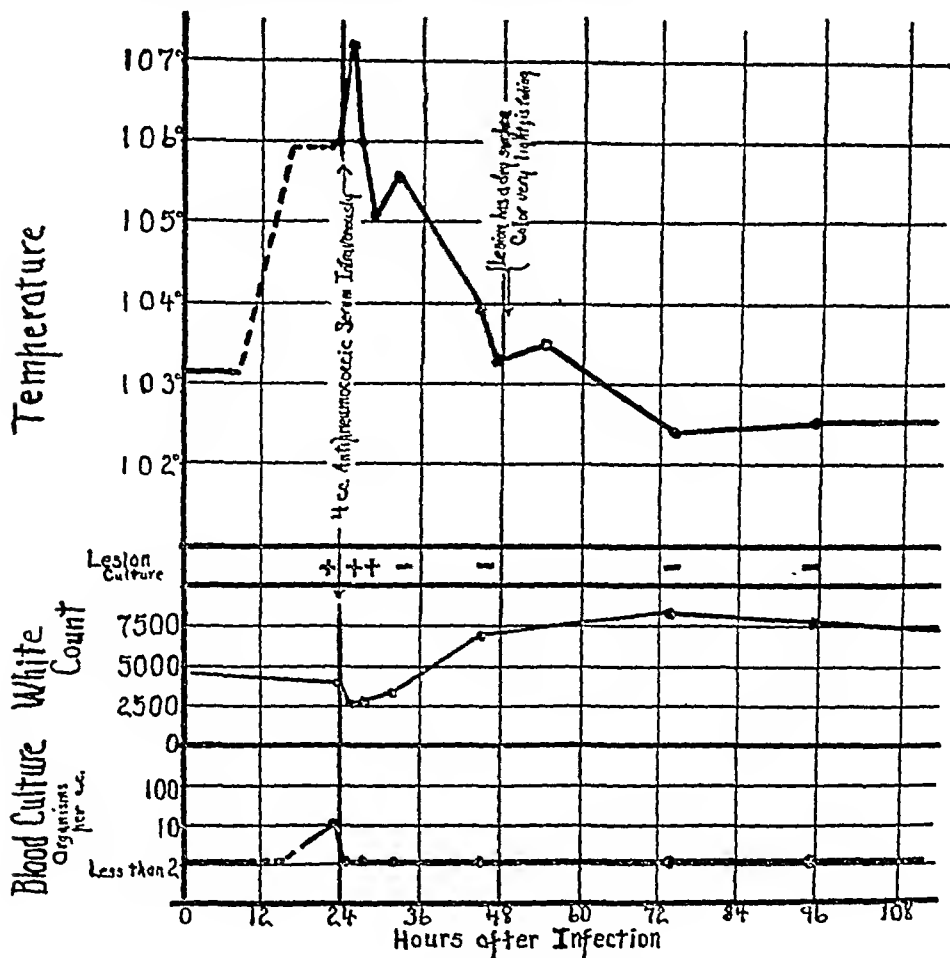
TEXT-FIG. 4.   This chart shows the results obtained by treatment of a typical case with an amount of serum (4 cc.) which represents an excess over the M.E.D. (3 cc. for this serum).   Weight of rabbit 1500 gm.   Time of treatment, 24 hours.

Response of Infected Rabbits to Specific Serum Therapy.

Early in the course of this study, we began to experiment with various types of therapy.   Since the intradermal infection of the rabbit represented a rather definite clinical symptom-complex, it was

reasoned that it might prove adaptable for (1) an analysis of the therapeutic action of specific sera, (2) for comparing the therapeutic values of various types of sera, and (3) for a method of standardization based on therapeutic value.
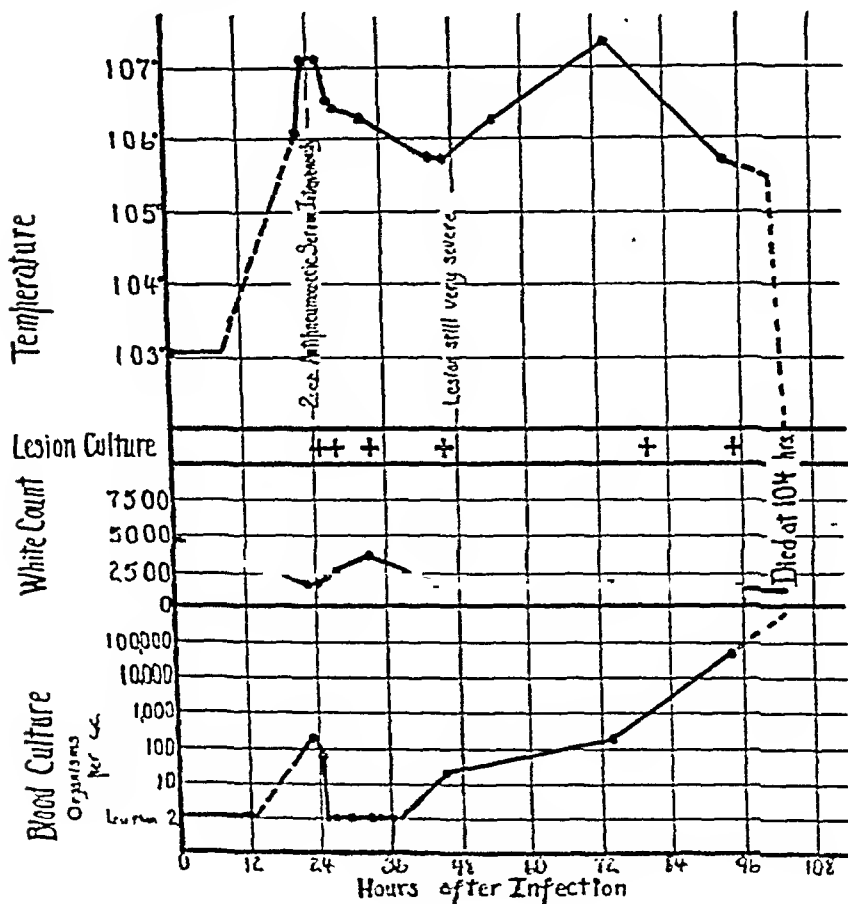
TEXT-FIG. 5. This chart shows the effect of treatment with an insufficient amount of serum. 2.0 cc. of a serum having a M.P.D. of 3.0 cc. were given at 24 hours. The only decided result is the temporary clearing of organisms from the blood stream.

We have not employed local therapy extensively, for, although antipneumococcic serum has a definite local protective effect in skin

areas in rabbits, it appears to have little influence if injected directly into an already active lesion.

Preliminary experiments demonstrated that prompt recovery could be brought about by the intravenous injection of a relatively large quantity of antipneumococcic serum provided that such injection be given early in the course of the disease. Further experiments were undertaken with the view of analysis of this therapeutic effect.

Text-fig. 4 shows the results obtained in a typical case by the intravenous injection at 24 hours following infection of a large amount (4 cc.) of antipneumococcic horse serum. The essential features of this therapeutic effect are: (1) the organisms immediately disappear from the blood and do not reappear, (2) the temperature begins to fall within a few hours and reaches a normal level within 24 hours, (3) organisms cannot be cultured from the local lesion after a period of a few hours following treatment, and (4) the skin lesion at first takes on a brighter color and then this color diminishes so that at 24 hours the area is very pale. At this time epidermal desquamation is also in evidence.

Experiments with a series of rabbits and graduated doses of serum show that beyond a certain point the excess of serum adds no value to that result which might be obtained with a definite minimum, this minimum being described as the minimal amount necessary to bring about the four decisive results given above. This definite value for any one serum is a relatively fixed quantity, other factors, such as time of treatment, being the same. This amount we designate as the minimal effective dose (M.E.D.).

If smaller amounts are administered it is found, depending upon the amount of serum, that there may be a temporary clearing of pneumococci from the blood stream but that this is not a permanent disappearance. In some instances (with doses that approach the M.E.D.) there may be a slight temporary depression of temperature and a considerable reduction in the bacterial content of the focal lesion. A chart of such a case is given in Text-fig. 5.

Observations on Active Immunization.

In the course of our work we have begun to apply this method of observation to the study of active immunization. While these studies

are being extended it is interesting in connection with the work reported above to note at this time the effects of a single injection of
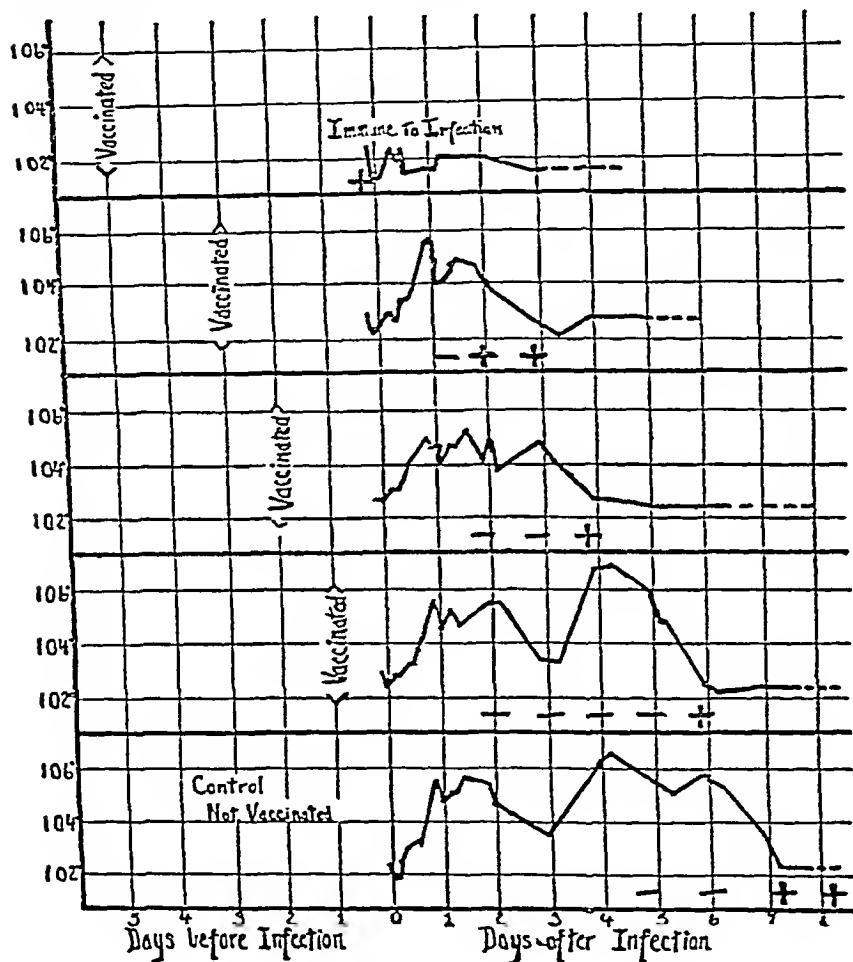
TEXT-FIG. 6. Temperature charts on a series of five rabbits, four of which had been vaccinated at various intervals prior to infection intradermally at 0 hour. In each case at the time of crisis the usual recessive features were noted in the local lesion. + and − indicate presence and absence of protective substances.

dead pneumococci on the development of the local lesion, the course of the disease, and especially the development of the sudden drop of

temperature which we have spoken of as the "crisis." This is illustrated in an experiment the results of which are shown in Text-fig. 6.

Text-fig. 6 indicates: (1) animals vaccinated by one injection of dead pneumococci 5 days before infection develop neither lesion nor general disease and protective substances are present on the 5th day after vaccination, (2) animals vaccinated 3 days before develop a definite, but rapidly resolving lesion and recover by crisis, again on the 5th day after vaccination. The protective bodies appeared at this time, and (3) with each day by which the interval between vaccination and infection is shortened the recovery by crisis and production of protective bodies are prolonged by 1 day (in some cases slightly more than 1 day).

It appears therefore that one injection of vaccine is followed with regularity in 5 or 6 days by a condition of immunity sufficient either to prevent infection, or, if infection precedes the lapse of this period, to bring about recovery within approximately 5 or 6 days after the administration of the dead bacteria.

Inference from this in regard to the possible usefulness of vaccine administration to cases of influenza or measles or in the early stages of pneumonia are obvious but will necessitate further study.

DISCUSSION.

The detailed discussion of these findings must await the results of experiments now being undertaken.

The chief value of this experimental condition is that it offers in a suitable laboratory animal a symptom-complex which is in many respects analogous to human lobar pneumonia and has the additional advantages of a visible and easily produced lesion. With this condition it has been possible to correlate some of the features involved in the local lesion and such more generalized factors as the febrile reaction, the bacteriemia, changes in leucocyte count, the development of protective substances, etc. While many of these correlations might have been predicted from our knowledge of lobar pneumonia, it nevertheless furnishes additional evidence of the similarity of this "dermal pneumonia" to that of true pneumonia.

Of particular interest in this condition is the temperature reaction; its features are an abrupt onset, the high level maintained during the

course of the disease, and the abrupt or critical fall. It has been shown that the febrile reaction is not primarily a factor of the bacteriemia but possibly of some unknown substance elaborated and absorbed from the active local lesion. Evidence is presented to show that the temperature onset coincides with the first appearance of the local lesion and that this temperature persists for a rather definite period of time quite independently of changes in the status of bacteriemia.

It has been shown that the critical fall in temperature is associated with the appearance of protective substances. We do not know what part such substances have in the actual recovery for it is certain that they do not appear before or during the temperature fall but do appear in maximum titer immediately after crisis. Such a crisis is preceded by a disappearance of organisms from the blood stream but not necessarily by their disappearance from the local lesion.

Changes in white cell count indicate that the leucocytes play a passive part in the disease in an untreated animal and that the polymorphonuclear elements are low in number during bacteriemia and conversely. The white count constitutes a definite prognostic sign in this respect only.

With this method it is possible to analyse carefully the result of therapy and to compare the effects from various therapeutic agencies. We have reported our observations in cases treated with the ordinary antipneumococcic horse serum. The study of passive immunization is being continued and we are now developing in detail a method for standardization of the therapeutic properties of serum, based on the observation as to the minimal effective dose for any one serum. It is hoped that such a method may offer a more satisfactory basis for measurement of therapeutic value than the present mouse protection titration.

SUMMARY.

1. The intradermal inoculation of rabbits with Type I pneumococci gives rise to a local lesion and a definite sequence of other events that offer many analogies to pneumococcus lobar pneumonia, and for this reason the condition is being employed for a resurvey of the subject of pneumococcic infection.

2. This symptom-complex has been described in detail particularly as to the development of the local lesion, the temperature reaction, the bacteriemia, the white count, the persistence of organisms in the lesion, and the development of protective substances.

3. A certain number of animals recover from this condition after a definite course and by a process spoken of as crisis. The events correlated with this crisis have been described.

4. If sufficient antipneumococcic serum is given intravenously at 24 hours, prompt recovery can be brought about. The essential points of this recovery have been established.

5. Within 5 days after a single vaccination with dead pneumococci the normal rabbit develops an immunity to infection. If the rabbit is vaccinated and then infected within the period necessary for the development of this immunity the course of the consequent disease is shortened in proportion to the interval between vaccination and infection.

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Drawing of a characteristic lesion at 30 hours following intradermal infection.

PLATE 2.

FIG. 2. A photograph of a cross-section of pneumococcic rabbit skin; low magnification. The original cross-dimension of this skin was 13.5 mm.

FIG. 3. Highly magnified detail of the section shown in Fig. 2. The structure of the phlegmon is shown, and at the upper right there is an injected lymphatic.
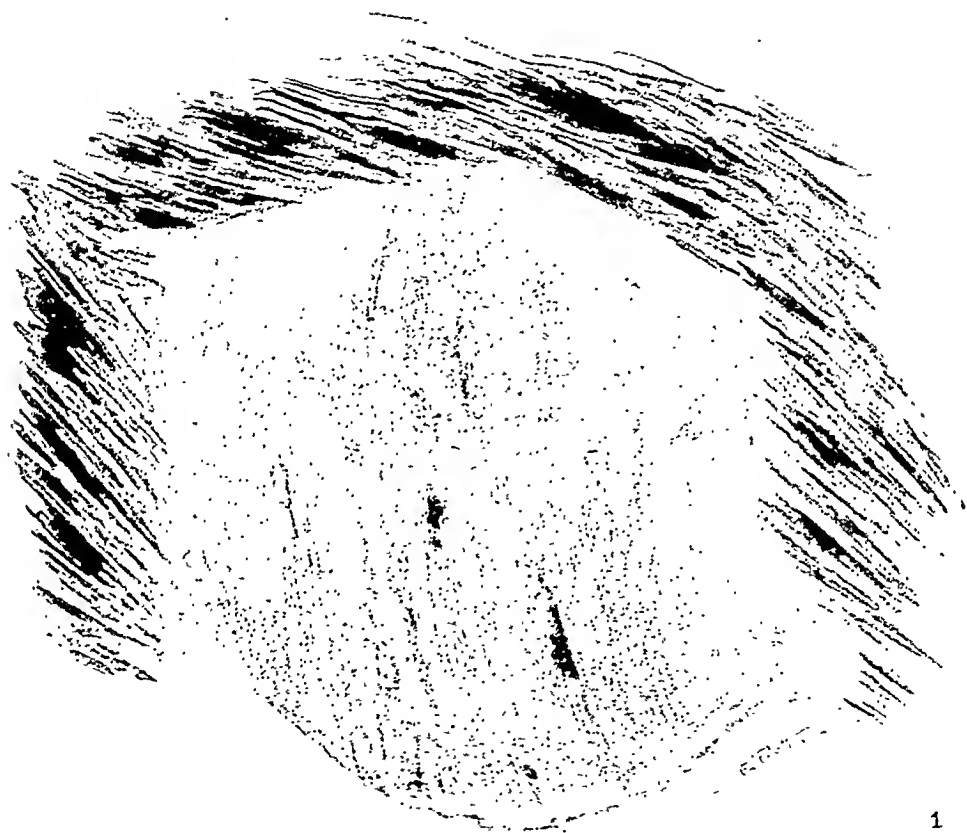
PLATE 1.

THE JOURNAL OF EXPERIMENTAL MEDICINE VOL. XLVIII.

1

CHOLESTEROL AND CHOLESTEROL ESTER CONTENT OF BOVINE COLOSTRUM.

By RICHARD E. SHOPE, M.D., and JOHN W. GOWEN, Ph.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Colostrum, the first milk secreted in a lactation period is distinguished by the quantitative relationship of many constituents from the milk of later lactation. It is richer in the albumin and globulin fractions of the protein and in the salts. It contains many cells frequently spoken of as milk corpuscles which contain nuclear material. The melting point of its fat is higher. Fox and Gardner (1) and Dorlencourt and Palfy (6) working with human milk have observed that that secreted early in lactation is richer in cholesterol than that secreted late in lactation.

In so far as the recent literature refers abundantly to the relationship existing between cholesterol or related compounds and vitamine D, this possible change in the cholesterol content from colostrum to milk is of particular interest in its relation to the antirachitic and growth functions associated with colostrum or milk. In conjunction with investigations designed to test the validity of this viewpoint, colostrum was drawn from three pure bred Jersey cows at 12 hour intervals, commencing at 12 hours after parturition. The sample of colostrum was a true sample of all the colostrum secreted, as the calf was removed from the dam before suckling, and colostrum was drawn only at these intervals when the cow was milked dry. Two of the cows, Nos. 232 and 235, were being treated with ultra-violet light and No. 237 was not. The animals were all protected from direct sunlight. The later samples of milk were obtained in the 4th and 8th months of lactation. Cholesterol was determined by the method of Bloor (2) for blood using double quantities of milk and double quantities of the alcohol ether extractive. Bloor and Knudson's method (3) was used in determining cholesterol bound as ester modifying it in the same way as for the total cholesterol analyses.

DISCUSSION.

The main fact brought out by this investigation is the large proportion of cholesterol in the first colostrum drawn as contrasted with that secreted in the next 12 hours.   The decline in the proportion of cholesterol secreted continues at an ever decreasing rate for the suc-

TABLE I.

Cow No.	Time after parturition that sample was drawn	Weight of sample	Total cho-lesterol	Cholesterol ester	
		kg.	*mg. per cent*	*mg. per cent*	*per cent*
235	12 hrs.		89	25	28
	24 "		53	14	27
Received ultra-violet light	36 "		34	7	19
	48 "		25	4	18
	Normal milk sample				
	3 mos.		17	6	36
	7 " a.m.		21	11	51
	7 " p.m.		23	6	25
232	12 hrs.	5.09	82	24	30
	24 "	5.72	38	8	22
Received ultra-violet light	36 "	7.54	36	7	20
	48 "	6.00	29	8	26
	Normal milk sample				
	3 mos.		19	4	25
	7 " a.m.		23	8	36
	7 " p.m.		23	3	13
237	12 hrs.	7.36	61	5	9
	24 "	3.50	46	5	11
Received no ul-tra-violet light	36 "	5.00	33	8	23
	48 "	5.09	29	4	15
	Normal milk sample				
	3 mos.		28	8	30
	7 " a.m.		19	12	64
	7 " p.m.		22	4	19

ceeding three periods of 12 hours each, when it reaches a level but little above that of the milk at 3 months.   This decline in the proportion of cholesterol represents also a true quantitative decline as the weights of the 12 hour colostrum samples remain fairly constant.   The level of cholesterol secretion is fairly constant for milk, as shown by compar-

ing the cholesterol in the milk at 3 and at 7 months. The significance of this decline in cholesterol is mathematically attested by the fact that Z is 1.6, while for $P = 0.05$, Z is only 0.6.[*] If this general trend in the cholesterol is compared with that of the other constituents in the colostrum, it is noted that the decline follows closely the secretion rates of the albuminous constituents up to the 48 hour period (4). After this time the albumin still continues to decline in value whereas the cholesterol remains fairly constant. A similar but less comparable decline in percentage amount occurs in the ash of the milk. The fat, casein and lactose show, if anything, a reverse tendency, rising in percentage rather than declining as lactation advances.

The total cholesterol per cent secreted by the cows receiving the ultra-violet light is larger than for the cow not receiving ultra-violet light for the first 12 hour period. Mathematically speaking, the difference is only suggestive, as the difference between ultra-violet and normal cows is less than 3 times its probable error.

The ester cholesterol of the colostrum appears to be quite variable. Cow 235 shows a decline comparable with that found for the total cholesterol. The other cows, especially for the 24, 36 and 48 hour periods, do not show the decline. The cows receiving ultra-violet light show slightly larger ester values than the cow not receiving light.

The morning and night variation of the cholesterol in 7 months milk indicates that normal Jersey milk contains about 22 mg. per cent of cholesterol. The amount of this total cholesterol bound as ester is twice as much in the morning milk as in the evening milk. This difference is significant as the value of P between the morning and night series is 0.03.

SUMMARY.

The total amount of cholesterol found in colostrum and milk is comparatively low. The amount of cholesterol found in colostrum declines at an ever decreasing rate as milk secretion develops until at 48 hours the cholesterol is nearly the same as that found in milk 3 months or 7 months after parturition. The morning milk differs from the evening milk in that the cholesterol bound as ester is greater in amount.

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[*] In calculating the significance of the decrease found against the normal variation, P is a direct measure of the significance of the data obtained. Z is a calculated constant from which P or probability is obtained (5).

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CHOLESTEROL AND CHOLESTEROL ESTER IN THE BLOOD SERUM OF CATTLE LATE IN PREGNANCY AND DURING THE EARLY LACTATION PERIOD.

By RICHARD E. SHOPE, M.D., AND JOHN W. GOWEN, PH.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

INTRODUCTION.

Chauffard, Laroche and Grigaut (1), Pribram (2) and others have observed that in the human being there is an increase in the cholesterol content of the blood throughout the latter half of pregnancy. After parturition and coincident with lactation a decrease in blood cholesterol results. The findings of Parhon and Parhon (3) on ducks and chickens indicate that in birds in the period just preceding laying there is an increase in the cholesterol content of the blood. During the egg-laying period there is a decrease in blood cholesterol. Baumann and Holly (4), using rabbits, found that, in pregnant animals, the concentration of cholesterol in the blood decreases from the 15th to 20th day after conception to reach the lowest values 1 to 4 days before parturition. The values rise again, irrespective of lactation, to the non-pregnant figures in 4 weeks after parturition.

The research herein reported was initiated for two reasons; the contrasting nature of the results on humans and birds as compared with those on rabbits, and the fact, which our previous investigations established (5), that the cholesterol in the colostrum period of mammary gland secretion is richest for the first secretion drawn, rapidly declining, as secretion continues, to a fairly constant level in the milk. The question therefore interests us as to whether these changes are due to an increase in blood cholesterol, or whether they are due to deposits of cholesterol in the udder during the dry period, or, third, to a greater secretory activity of the gland for this substance as contrasted with the other materials making up colostrum.

TABLE I.

Cow No.	Date and days in relation to parturition	Total serum cholesterol	Serum cholesterol ester	
	1927	*mg. per cent*	*mg. per cent*	*per cent*
45	Mar. 26 (30 days ante partum)	152	66	44
	Apr. 7 (18 " " ")	149	81	54
	Apr. 16 (9 " " ")	113	68	60
	Apr. 25, calved			
	Apr. 27 (2 days post partum)	100	54	54
	May 2 (7 " " ")	120	75	63
	May 6 (11 " " ")	114	84	74
	May 11 (16 " " ")	141	97	69
	May 16 (21 " " ")	162	102	63
	June 7 (43 " " ")	203	Lost	
	June 17 (53 " " ")	170	139	82
	June 27 (63 " " ")	211	119	56
141	Mar. 17 (39 days ante partum)	87	45	51
	Mar. 26 (30 " " ")	93	53	58
	Apr. 7 (18 " " ")	98	64	65
	Apr. 16 (9 " " ")	94	71	75
	Apr. 25, calved			
	Apr. 27 (2 days post partum)	94	54	51
	May 2 (7 " " ")	121	61	50
	May 6 (11 " " ")	122	77	63
	May 11 (16 " " ")	127	76	60
	May 16 (21 " " ")	119	83	70
	June 7 (43 " " ")	174	Lost	
	June 17 (53 " " ")	187	134	72
	June 27 (63 " " ")	181	139	77
47	Mar. 17 (32 days ante partum)	110	64	58
	Mar. 26 (23 " " ")	130	68	52
	Apr. 7 (11 " " ")	130	77	59
	Apr. 16 (2 " " ")	123	85	69
	Apr. 18, calved			
	Apr. 20 (2 days post partum)	104	76	73
	Apr. 25 (7 " " ")	114	76	67
	Apr. 30 (12 " " ")	117	79	68
	May 6 (18 " " ")	133	90	67
	May 11 (23 " " ")	130	102	78
	May 16 (28 " " ")	152	110	72
	June 7 (50 " " ")	175	Lost	
	June 17 (60 " " ")	153	133	87
	June 27 (70 " " ")	183	89	48

TABLE I—*Concluded.*

Cow No.	Date and days in relation to parturition	Total serum cholesterol	Serum cholesterol ester	
	1927	*mg. per cent*	*mg. per cent*	*per cent*
185	Mar. 17 (30 days ante partum)	163	96	59
	Mar. 26 (21 " " ")	139	95	68
	Apr. 7 (9 " " ")	130	93	71
	Apr. 16, calved			
	Apr. 18 (2 days post partum)	139	41	29
	Apr. 22 (6 " " ")	133	93	69
	Apr. 26 (10 " " ")	125	79	63
	Apr. 30 (14 " " ")	119	79	66
	May 6 (20 " " ")	125	93	74
	May 11 (25 " " ")	125	96	76
	May 16 (30 " " ")	133	76	57
	June 7 (52 " " ")	192	Lost	
	June 17 (62 " " ")	189	149	79
	June 27 (72 " " ")	189	107	56

EXPERIMENTAL.

Blood from four cows, 240, 241, 247 and 252 days pregnant, was obtained. The animals were bled from the jugular vein at intervals of a few days as given in Table I. Serum cholesterol was determined by the method of Bloor (6), and cholesterol ester by the method of Bloor and Knudson (7). The results obtained are given in Table I.

DISCUSSION.

Three very definite alterations occur in the cholesterol and cholesterol ester content of cow serum late in pregnancy and during the ensuing lactation period. First, the non-lactating period of late pregnancy is characterized by a serum low in both cholesterol and cholesterol ester. Secondly, 2 days post partum there is a further decrease in serum cholesterol ester and usually also in total serum cholesterol. Thirdly, during the early lactation period there is a gradual rise in both total serum cholesterol and serum cholesterol ester to reach a rather constant level sometime between 40 and 50 days post partum. The general trend of the serum cholesterol values is highly significant, mathematically speaking, as the value of Z is 0.97 where the value of
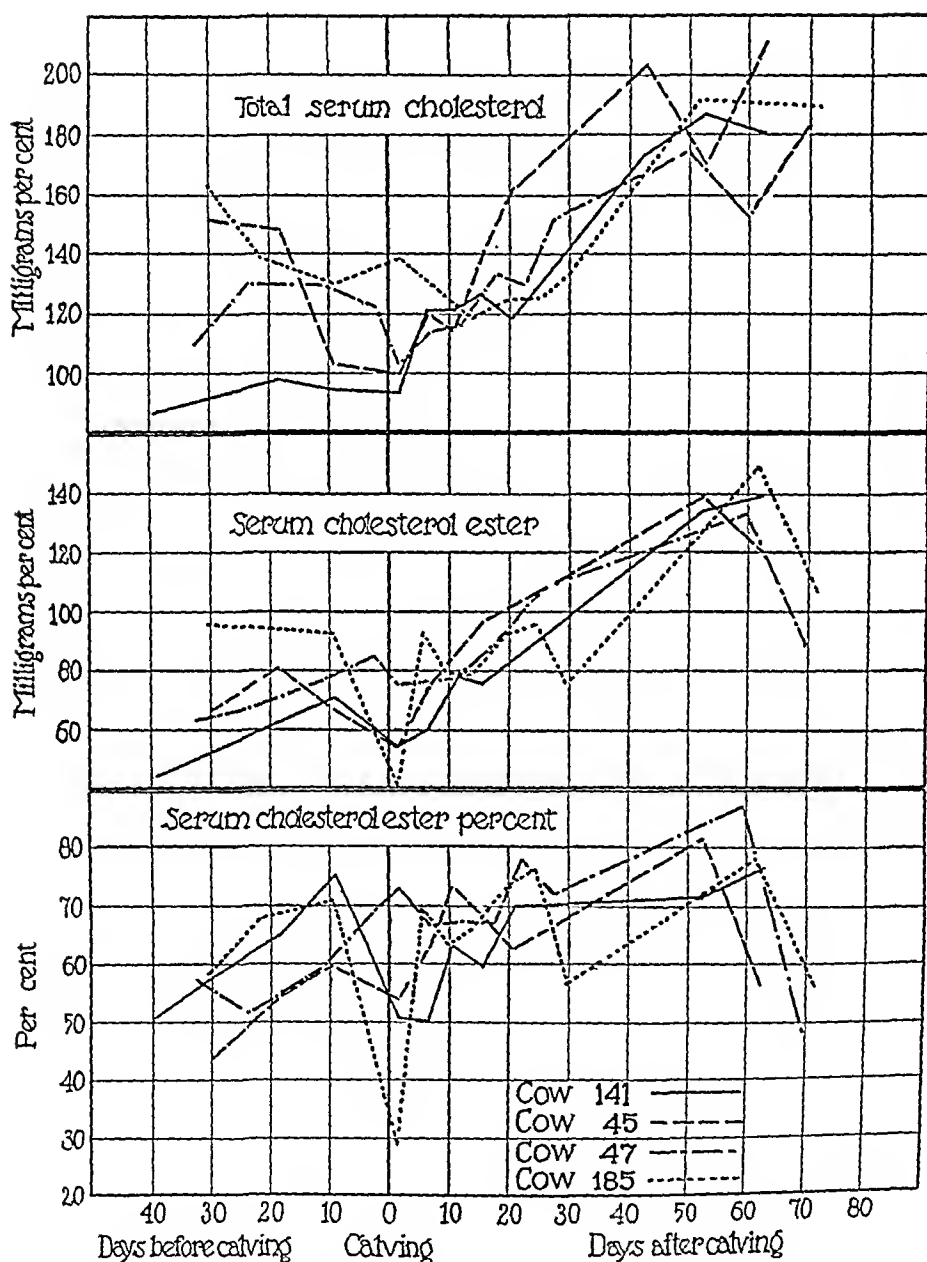
Fig. 1. Relation between pregnancy, parturition and lactation of the serum cholesterol and cholesterol ester in dairy cattle.

Z for $P = 0.05$ is only 0.39.[*]   These changes may be most clearly visualized from Fig. 1.

There is some evidence tending to show a slight individuality in the serum cholesterol values of the cows making up the experiment.   The value of Z for the four cows is 0.58, whereas that for $P = 0.05$ is 0.54, or the individuality of the cows in their serum cholesterol values is just over the border line of significance.   The results therefore possibly show that the effect of physiological factors on serum cholesterol values is itself influenced by the individuality of the cow.

The cholesterol values of the blood serum as herein indicated show a trend opposite to that found for colostrum and milk as presented elsewhere (5), for instead of rising after parturition the cholesterol of colostrum and of milk declines.   The cholesterol values of blood serum rise to a maximum at about 40 to 50 days.   This rise corresponds in time to the rise taking place in the milk production and the attendant rise of the milk solids for most cows.   It corresponds further to the period of more than ordinarily rapid drain on the body stored materials.   This is particularly true of dairy cattle for which the aim of the breeder has always been the production of cows capable of a maximum milk yield.

The work herein described agrees with the facts found for rabbits.

SUMMARY AND CONCLUSIONS.

1. The total cholesterol and cholesterol ester of bovine blood serum during the "dry" period of late pregnancy is low.

2. Following parturition the total cholesterol and cholesterol ester of bovine blood serum show a still greater, very transient decrease followed by a gradual rise to reach a rather constant level 40 to 50 days post partum.   This lactation level is very much higher than the "dry" level.

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[*] In calculating the significance of the general trend of serum cholesterol against the normal variation, P is a direct measure of the data obtained. Z is simply a calculated constant from which P or probability is obtained (8).

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INFLUENCE OF LIGHT ENVIRONMENT ON THE GROWTH AND NUTRITION OF NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

By WADE H. BROWN, M.D.

With the Collaboration of Marion Howard.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 3.

It was shown in a previous paper (1) that the calcium and inorganic phosphorus in the blood of normal rabbits is subject to periodic variations and that among animals living in the laboratory there is also a tendency toward a progressive increase in calcium, as was first noted by Grant and Gates (2), and a more decided decrease in inorganic phosphorus of uncertain duration and extent with consequent variations in the equilibrium between the two substances. It appeared that several factors might contribute to the production of these variations among which were increasing age, adjustment to cage life, and changing conditions of light environment. The influence of light environment on the calcium and inorganic phosphorus content of the blood has been tested experimentally, but before proceeding with the analysis of the results obtained, it seems desirable to direct attention to some other observations which were made on the same animals, as these observations afford some interesting examples of the manner and extent to which functional activities and chemical equilibria may be affected by prolonged exposure to a light environment of a given type.

The object of the present paper is to report the results of a comparison of changes in body weight of normal rabbits living under different environmental conditions with especial reference to the action of neon light and to the existence of a relation between the nutritive status of an animal and the chemical composition of the blood under the conditions employed. This latter aspect of the subject will not be developed in this paper further than to point out a few instances in which there was some evidence of the occurrence of coordinate variations in body

weight and blood calcium. This phase of the problem will be presented in a subsequent paper.

Material and Methods.

The results to be reported in this paper represent one phase of a series of observations which were made on rabbits living under conditions which differed only with respect to the light environment. The observations made included records of weight, determinations of calcium and inorganic phosphorus from the same sample of blood, a study of the proliferative activity of hair follicles as indicated by the rate and extent of the regeneration of hair over shaved areas, and a comparison of the weights of organs at the conclusion of the experiments. This paper will be limited to a consideration of the results obtained for body weights with a brief reference to the blood findings in certain groups of animals.

The effects of three environmental conditions were compared. One group of animals, which may be designated as the controls, was kept in a well lighted (sunlight), well ventilated room with a southern exposure. The room was heated during cold weather and an effort was made to maintain a temperature of 60–70°F. (thermostatic regulation).

A second group of rabbits was kept in a room from which all light was excluded. Other living conditions were the same as those of the first group. During the course of the experimens, these animals received some light from two sources. For a brief period each day, a 30 watt Mazda lamp was used in this room for cleaning cages, feeding, and making the necessary observations. From time to time, the animals were also brought into the laboratory for bleeding and were thus exposed to diffuse filtered sunlight for a period which rarely exceeded an hour per week.

A third group of animals, the light group, was kept in a room similar to the others, with sunlight excluded. This room was lighted continuously by 20 standard neon tubular lights (crown glass), 6 feet in length and 5/8 inches in diameter. The tubes were mounted in an upright position on a rectangular frame in the center of the room, parallel with and facing the cages at a distance of approximately 3½ feet. The lights were operated on a current of 25 milliamperes at 8000 volts, to a bank of 3 lamps in series.

The light produced by neon Geissler tubes varies from a rose red to a bluish pink color depending upon the condition and the operation of the tube. The heat produced is very slight.

A spectrogram of neon light in crown glass is shown in Fig. 1, for which we are indebted to Dr. F. L. Gates. The rays transmitted are concentrated in two regions with faint bands intervening. The shortest rays lie between 3370 and 3620 Ångström units. The strongest of these are in the region of 3460 to 3480. The greater part of the light produced is of very long wave-length and is composed of rays from about 5800 to the lower limit of registration at 7600 Ångströms. The vertical illumination at the front of the cages varied between 21 or 23 foot candles at the ends of the rack and 54 at the center with a mean of 34.3 foot candles. No attempt was made to measure the energy delivered by this equipment

as it was perfectly obvious that such measurements would serve no particular purpose in the present instance.

We wish also to emphasize the fact that the animals used in these experiments were given no special preparation to facilitate the action of light such as clipping or shaving of hair.

Observations were made on three groups of normal male rabbits living under the conditions described. At the beginning of the experiments, the animals were 8 to 10 months old; they were sexually mature but had not attained their full growth. In each case, the animals for a given experiment were assembled and divided into three comparable subgroups according to type or breed, age, and weight. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage. They were weighed before being fed and, as far as possible, on the same day of each week throughout the period of observation.

Group I contained 15 albino rabbits, Group II, 15 black rabbits, and Group III, 15 gray, brown, or black rabbits. Groups I and II were placed under observation October 1, 1926 and were followed under control conditions until October 22; they were then placed in their respective experimental quarters and observations continued until May 18, 1927.

Observations on Group III began October 1, 1926. On February 8, 1927, the light and dark divisions of this group were interchanged so that animals which had been living in an environment of neon light for 4 months were placed in darkness and *vice versa*. This condition was maintained until May 4, 1927 when the two groups were again interchanged or restored to their original environment and observations continued until June 8, 1927.

The results obtained from the 3 experiments described have been supplemented by results from observations on two other groups of 10 rabbits each living under control conditions. These animals were grays and browns and were slightly younger than those in the regular experiments. The observations on one group (Group IV) began January 1, 1927 and on the other (Group V) March 1, 1927, and were continued, in both cases, until July 9, 1927.

The results of the study of body weight, as affected by light environment, are assembled in Tables I to XI. These tables contain mean values for the weight of given groups of animals at the time they were assembled, which is designated as the initial weight, and for consecutive periods of observation. In order to minimize the error due to unavoidable variations in the gastro-intestinal contents, the mean values have been smoothed by the formula $\dfrac{A + 2B + C}{4}$ and the smoothed value, rather than the actual mean, has been used as the basis of estimating gain or loss. The figures given for the gain in weight represent the difference between the initial weight and succes-

sive smoothed values.   In order to reduce all expressions of weight relations to a comparable basis, the gain or loss has been converted into per cent of the initial weight, and these values are used for plotting the curves in Text-figs. 1 to 6.

RESULTS.

The results of the experiments are summarized in Tables I to XI and Text-figs. 1 to 6.

TABLE I.

Group I Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2319			
Oct. 7	2350	2327	8	0.34
" 23	2290	2321	2	0.09
" 30	2355	2358	39	1.6S
Nov. 6	2430	2399	S0	3.45
" 13	2380	2390	71	3.06
" 20	2370	2376	57	2.46
" 27	2385	2386	67	2.89
Dec. 4	2405	2396	77	3.32
" 11	2390	2394	75	3.23
" 18	2390	2393	74	3.19
" 29	2400	2433	114	4.92
1927				
Jan. 8	2540	2513	194	8.37
" 15	2570	2561	242	10.44
" 22	2565	2580	261	11.25
" 29	2620	2610	291	12.55
Feb. 5	2635	2623	314	13.54
" 11	2600	2608	2S9	12.46
" 19	2595	2589	270	11.64
" 25	2565	2563	244	10.52
Mar. 5	2525	2528	209	9.01
" 12	2495	2515	196	8.45
" 19	2545	2536	217	9.36
" 26	2560	2534	215	9.27
Apr. 2	2470	2468	149	6.43
" 9	2370	2453	134	5.78
" 16	2600	2550	231	9.96
" 23	2631	2619	300	12.94
" 29	2613	2625	306	13.20
May 7	2644	2644	325	14.01
" 14	2675	2666	347	14.96
" 19	2669			

TABLE II.

Group I Light — Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	gm.	gm.	gm.	per cent
Initial	1955			
Oct. 7	1912	1964	9	0.46
" 23	2075	2054	99	5.06
" 30	2155	2139	184	9.41
Nov. 6	2170	2209	254	12.99
" 13	2340	2311	356	18.21
" 20	2395	2390	435	22.25
" 27	2430	2406	451	23.07
Dec. 4	2370	2408	453	23.17
" 11	2460	2433	478	24.45
" 18	2440	2465	510	26.09
" 29	2520	2479	524	26.80
1927				
Jan. 8	2435	2484	529	27.06
" 15	2545	2523	568	29.05
" 22	2565	2555	600	30.00
" 29	2545	2553	598	30.59
Feb. 5	2555	2558	603	30.84
" 11	2575	2599	644	32.94
" 19	2690	2660	705	36.06
" 25	2685	2691	739	37.80
Mar. 5	2715	2704	749	38.31
" 12	2700	2725	770	39.39
" 19	2785	2748	793	39.75
" 26	2720	2765	810	41.43
Apr. 2	2835	2780	825	42.20
" 9	2730	2764	809	40.55
" 16	2760	2781	826	42.25
" 23	2875	2840	885	45.27
" 29	2850	2850	895	45.28
May 7	2825	2835	883	45.17
" 14	2850	2833	878	44.91
" 19	2805			

TABLE III.

Group I Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2150			
Oct. 7	2160	2171	20	0.98
" 23	2215	2221	71	3.30
" 30	2295	2309	159	7.40
Nov. 6	2430	2380	230	10.70
" 13	2365	2403	253	11.77
" 20	2450	2375	225	10.47
" 27	2235	2315	165	7.67
Dec. 4	2340	2353	203	9.44
" 11	2495	2453	303	14.09
" 18	2480	2455	300	13.95
" 29	2363	2433	283	13.16
1927				
Jan. 8	2525	2443	293	13.63
" 15	2360	2408	258	12.00
" 22	2385	2363	213	9.91
" 29	2320	2380	230	10.70
Feb. 5	2495	2430	280	13.02
" 11	2410	2433	283	13.16
" 19	2415	2413	263	12.23
" 25	2410	2446	296	13.77
Mar. 5	2550	2484	334	15.53
" 12	2425	2483	333	15.49
" 19	2530	2498	348	16.19
" 26	2505	2509	359	16.70
Apr. 2	2495	2501	351	16.33
" 9	2510	2481	331	15.40
" 16	2410	2479	329	15.30
" 23	2585	2508	358	16.65
" 29	2450	2491	341	15.86
May 7	2480	2485	335	15.58
" 14	2530	2499	349	16.23
" 19	2455			

TABLE IV.

Group II Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2430			
Oct. 7	2480	2486	56	2.30
" 23	2555	2555	125	5.14
" 30	2628	2607	177	7.28
Nov. 6	2615	2606	176	7.24
" 13	2565	2585	155	6.38
" 20	2595	2596	166	6.83
" 27	2630	2624	194	7.98
Dec. 4	2640	2646	216	8.89
" 11	2675	2668	238	9.79
" 18	2680	2680	250	10.29
" 29	2685	2690	260	10.70
1927				
Jan. 8	2710	2710	280	11.52
" 15	2735	2756	326	13.42
" 22	2845	2825	395	16.26
" 29	2875	2865	435	17.90
Feb. 5	2865	2849	419	17.24
" 11	2790	2818	388	15.97
" 19	2825	2798	368	15.14
" 25	2750	2768	338	13.97
Mar. 5	2745	2745	315	12.96
" 12	2740	2756	326	13.42
" 19	2800	2784	354	14.57
" 26	2795	2794	364	14.93
Apr. 2	2785	2766	336	13.83
" 9	2700	2716	286	11.77
" 16	2680	2686	256	10.53
" 23	2685	2703	273	11.23
" 29	2760	2758	328	13.50
May 7	2825	2821	391	16.09
" 14	2875	2829	399	16.42
" 19	2740			

TABLE V.

Group II Light—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2405			
Oct. 7	2455	2426	21	0.87
" 23	2390	2425	20	0.83
" 30	2465	2465	60	2.49
Nov. 6	2540	2559	154	6.40
" 13	2690	2663	258	10.73
" 20	2730	2735	330	13.72
" 27	2790	2764	359	14.93
Dec. 4	2745	2795	390	16.22
" 11	2900	2878	473	19.67
" 18	2966	2957	552	22.95
" 29	2994	2946	541	22.49
1927				
Jan. 8	2830	2896	491	20.42
" 15	2930	2925	520	21.62
" 22	3010	2978	573	23.83
" 29	2961	2977	572	23.78
Feb. 5	2975	2978	573	23.83
" 11	3005	3023	618	25.70
" 19	3115	3083	678	28.19
" 25	3100	3105	700	29.11
Mar. 5	3105	3094	689	28.65
" 12	3065	3091	686	28.52
" 19	3130	3096	691	28.73
" 26	3060	3095	690	28.69
Apr. 2	3130	3098	693	28.81
" 9	3070	3081	676	28.11
" 16	3055	3086	681	28.32
" 23	3165	3129	724	30.10
" 29	3130	3134	729	30.31
May 7	3110	3135	730	30.35
" 14	3190	3150	745	30.98
" 19	3110			

TABLE VI.

Group II Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2100			
Oct. 7	2150	2134	34	1.62
" 23	2135	2151	51	2.43
" 30	2185	2206	106	5.05
Nov. 6	2320	2281	181	8.62
" 13	2300	2333	233	11.10
" 20	2410	2311	211	10.05
" 27	2125	2234	134	6.38
Dec. 4	2275	2269	169	8.05
" 11	2400	2365	265	12.62
" 18	2385	2385	285	13.57
" 29	2370	2393	293	13.95
1927				
Jan. 8	2445	2389	289	13.76
" 15	2295	2339	239	11.38
" 22	2320	2303	203	9.67
" 29	2275	2320	220	10.48
Feb. 5	2410	2365	265	12.62
" 11	2365	2373	273	13.00
" 19	2350	2346	246	11.71
" 25	2320	2360	260	12.38
Mar. 5	2450	2390	290	13.81
" 12	2340	2413	313	14.90
" 19	2520	2460	360	17.14
" 26	2460	2473	373	17.76
Apr. 2	2450	2448	348	16.57
" 9	2430	2414	314	14.95
" 16	2345	2393	293	13.95
" 23	2450	2405	305	14.52
" 29	2375	2404	304	14.48
May 7	2415	2418	318	15.14
" 14	2465	2436	336	16.00
" 19	2400			

TABLE VII.

Group III Control—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2200			
Oct. 15	2260	2315	115	5.23
Nov. 24	2540	2476	276	12.55
Dec. 29	2555	2558	358	16.27
1927				
Jan. 8	2580	2570	370	16.82
" 15	2565	2570	370	16.82
" 22	2570	2570	370	16.82
" 29	2575	2573	373	16.95
Feb. 5	2575	2581	381	17.39
" 11	2600	2603	400	18.18
" 19	2635	2635	435	19.77
" 25	2670	2678	478	21.73
Mar. 9	2735	2711	511	23.23
" 12	2705	2725	525	23.86
" 19	2755	2744	544	24.73
" 26	2760	2765	576	26.18
Apr. 2	2785	2780	580	26.36
" 9	2790	2791	591	26.86
" 16	2800	2806	606	27.55
" 23	2835	2820	620	28.18
" 30	2810	2789	589	26.77
May 7	2700	2738	538	24.45
" 14	2740	2751	551	25.05
" 18	2825	2796	596	27.09
" 25	2795	2779	579	26.32
June 3	2700	2723	523	23.77
" 8	2695			

TABLE VIII.

Group III Light—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2169			
Oct. 15	2288	2266	97	4.47
" 23	2319	2336	167	6.70
" 30	2419	2399	230	10.60
Nov. 6	2438	2488	319	14.71
" 13	2656	2603	434	20.01
" 20	2663	2672	503	23.19
" 27	2706	2694	525	24.20
Dec. 4	2700	2711	542	24.99
" 11	2738	2725	556	25.63
" 18	2725	2758	589	27.16
" 29	2844	2780	611	28.17
1927				
Jan. 8	2705	2770	601	27.71
" 15	2830	2815	646	29.78
" 22	2895	2873	704	32.46
" 29	2870	2881	712	32.83
Feb. 5	2890	2879	710	32.73
" 11	2965	2886	717	33.06
" 18	2925	2904	735	33.89
" 25	2900	2928	759	34.99
Mar. 5	2985	2944	775	35.73
" 12	2905	2969	800	36.88
" 19	3080	3041	872	40.20
" 26	3100	3074	905	41.72
Apr. 2	·3015	3019	850	39.19
" 9	2945	2989	820	37.81
" 16	3050	2996	827	38.13
" 23	2940	2954	785	36.19
" 30	2885	2924	755	34.81
May 7	2985	2974	805	37.11
" 14	3040	3023	854	39.37
" 21	3025	3034	865	39.88
" 27	3045	3005	836	38.54
June 7	2905	2945	776	35.78
" 9	2925			

TABLE IX.

Group III Dark—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1926	*gm.*	*gm.*	*gm.*	*per cent*
Initial	2220			
Oct. 15	2220	2251	31	1.40
" 23	2345	2334	114	5.14
" 30	2425	2440	220	9.91
Nov. 6	2565	2533	313	14.10
" 13	2575	2580	360	16.22
" 20	2605	2568	348	15.68
" 27	2490	2533	313	14.10
Dec. 3	2545	2555	335	15.09
" 11	2640	2612	392	17.66
" 18	2625	2614	394	17.75
" 29	2565	2571	351	15.81
1927				
Jan. 8	2530	2566	346	15.59
" 15	2640	2595	375	16.89
" 22	2570	2584	365	16.40
" 29	2555	2581	361	16.26
Feb. 5	2645	2608	388	17.48
" 11	2585	2605	385	17.34
" 19	2610	2606	386	17.39
" 25	2620	2638	418	18.83
Mar. 5	2700	2686	466	20.99
" 12	2725	2726	506	22.79
" 19	2755	2746	526	23.69
" 26	2750	2768	548	24.68
Apr. 2	2815	2789	569	25.63
" 9	2775	2799	579	26.08
" 16	2830	2849	629	28.33
" 23	2960	2925	705	31.76
" 29	2950	2945	725	32.66
May 7	2920	2940	720	32.43
" 14	2970	2928	708	31.89
" 21	2850	2850	630	28.38
" 28	2730	2806	586	26.40
June 7	2915	2811	591	26.62
" 9	2685			

TABLE X.

Group IV—Body Weights.

Date	Group mean	Smoothed mean	Net gain	Net gain
1927	*gm.*	*gm.*	*gm.*	*per cent*
Initial	1942			
Jan. 8	1961	1963	21	1.08
" 15	1986	1990	48	2.47
" 22	2028	2026	84	4.33
" 29	2061	2058	116	5.97
Feb. 5	2081	2085	143	7.36
" 11	2117	2127	185	9.53
" 19	2192	2166	224	11.53
" 25	2161	2182	240	12.36
Mar. 5	2213	2204	262	13.49
" 12	2230	2225	283	14.57
" 19	2225	2245	303	15.60
" 26	2300	2278	336	17.30
Apr. 2	2286	2288	346	17.82
" 9	2280	2266	324	16.68
" 16	2218	2244	302	15.55
" 23	2260	2255	313	16.12
" 29	2283	2289	347	17.87
May 7	2328	2331	389	20.03
" 14	2383	2362	420	21.63
" 27	2353	2362	420	21.63
June 3	2358	2337	395	20.34
" 18	2278	2329	387	19.93
" 25	2403	2359	417	21.47
July 1	2350	2377	435	22.40
" 9	2405			

TABLE XI.

Group V—Body Weights.

| Date	Group mean	Smoothed mean	Net gain	Net gain
1927	gm.	gm.	gm.	per cent
Initial	2075			
Mar. 23	2265	2201	126	6.07
Apr. 2	2198	2205	130	6.27
" 9	2160	2167	92	4.43
" 16	2150	2146	71	3.42
" 23	2125	2148	73	3.52
" 29	2190	2185	110	5.30
May 7	2223	2235	160	7.71
" 14	2283	2268	193	9.30
" 21	2273	2281	206	9.93
" 27	2295	2295	220	10.60
June 3	2315	2311	236	11.37
" 10	2320	2315	240	11.57
" 17	2305	2334	259	12.48
" 25	2405	2376	301	14.51
July 1	2388	2416	341	16.43
" 9	2482			
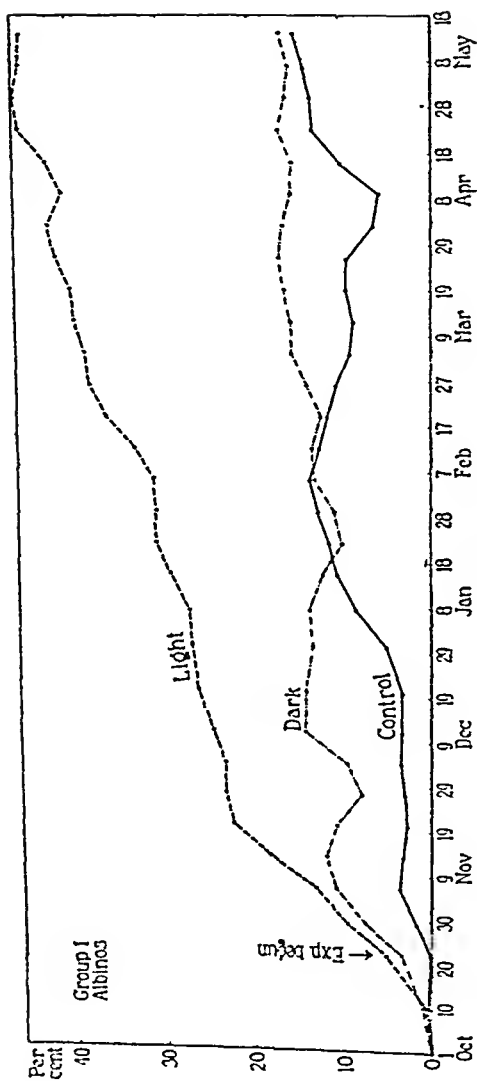
Text-Fig. 1.
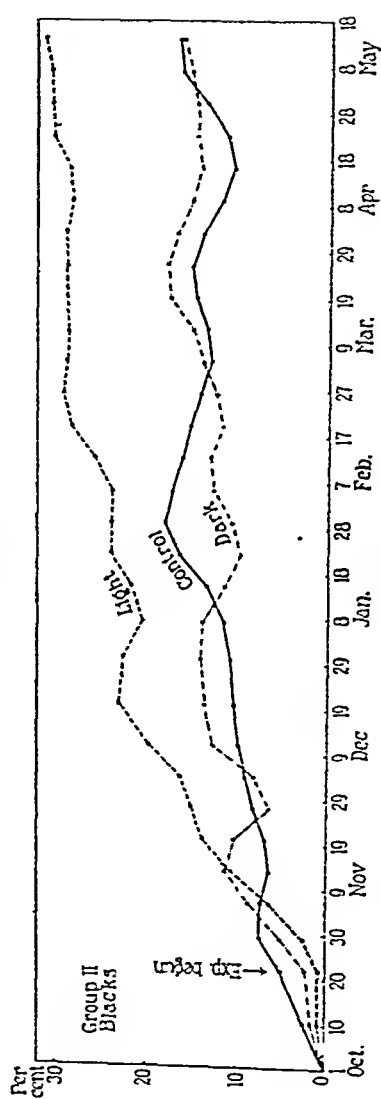
Text-Fig. 2.
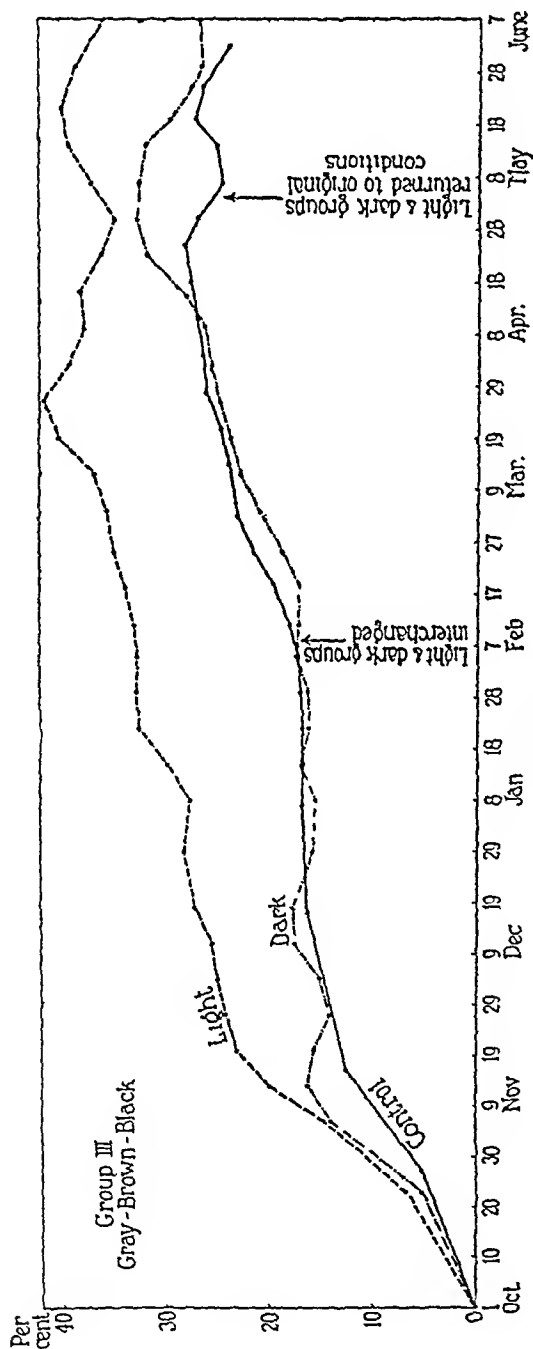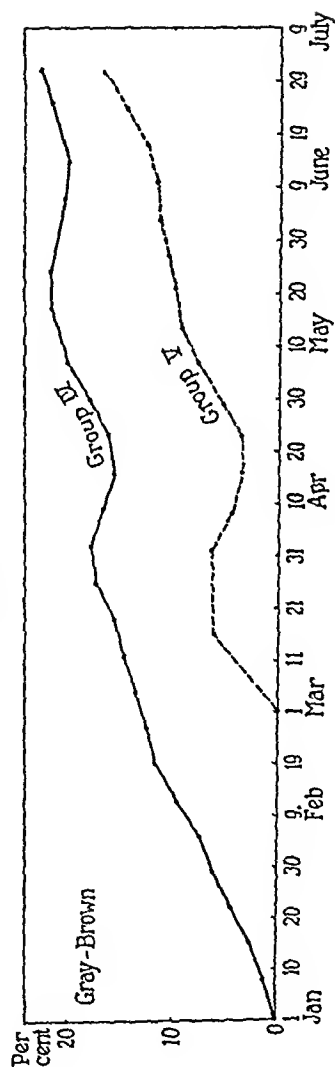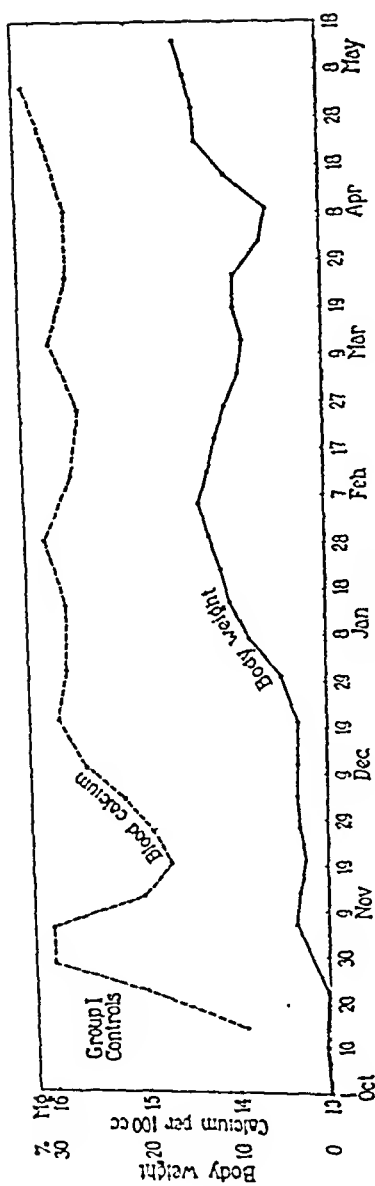
Text-Fig. 3.
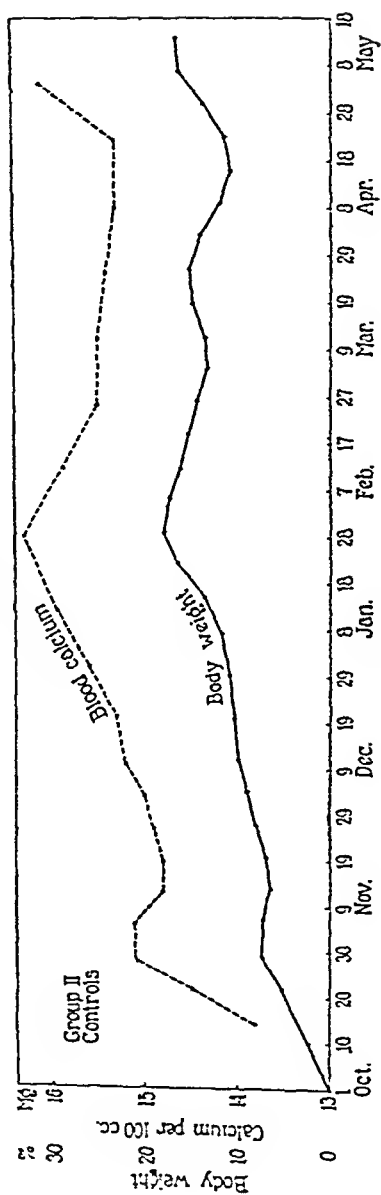
Text-Fig. 4.

TEXT-FIG. 5

TEXT-FIG. 6

DISCUSSION AND CONCLUSIONS.

From an examination of the text-figures, it is evident that the animals exposed to neon light showed a greater gain in weight than those receiving diffuse, filtered sunlight or living in darkness. It should be noted also that of the three groups of rabbits living in an environment of neon light, the greatest gain was made by the white animals and that the black animals gained the least which is the reverse of the condition shown by control and dark groups. Theoretically, the black animals might be expected to show the greatest gain as the animals of this group were of a larger breed (Flemish crosses), and in our experience rabbits of this type gain more under the usual laboratory conditions than the common albino, as was true in the present instance. In view of these facts, the gain made by the white animals under the influence of neon light is especially significant.

The differences between groups may be brought out more clearly by a tabulation of the maximum gain shown by each group in per cent of the initial weight. For the purpose of this comparison, the results for Group III are divided. The first column gives the gain prior to the interchange of light and dark animals while the second gives the maximum gain irrespective of changes in environment. The results on this basis are as follows:

Gain in Weight According to Groups.

	Group I	Group II	Group III	
Light	45.78	30.98	32.73	41.72
Dark	16.70	17.76	17.66	32.43
Control	14.96	17.90	17.39	28.18

Viewed from this standpoint, it will be seen that the gain in weight of the albino rabbits (Group I) receiving neon light was approximately 3 times that of the dark and control divisions of this group; in the case of the black animals and the dark colored animals of Group III, it was nearly twice as great. A further point of interest that is brought out by this table is the agreement of the results for animals of comparable or different types under a given condition. Thus, if we consider the first results for Group III, the agreement between Groups II and III

is very close for a given environmental condition. These two groups are comparable as both were composed of dark colored animals, while Group I was distinctly different and the results are of a different order with the exception of animals living in the dark; the difference in this case is very slight as might be expected.

Considering the effects of the change in light environment of the animals of Group III, it will be seen by reference to Text-fig. 3 that at the time this change was made the weights of light, dark, and control animals were in a state of virtual equilibrium, and that following the change, the weights of all three groups began to increase at nearly the same rate. This situation was maintained for about 5 weeks when the animals that had been transferred from light to dark showed a more rapid increase for a brief period, followed by a decided loss in weight which continued up to the time they were returned to the light.

Meantime, the weights of the animals which had been transferred from dark to light continued to increase at a rate only slightly greater than that of the controls, making a gain of approximately 9.00 per cent during a period of about 2 months as compared with practically no gain during the 4 months preceding. Then as the weights of the light animals decreased, those of the dark animals increased at a more rapid rate—adding 6.50 per cent to their weight in 3 weeks, while the light animals lost 7.00 per cent. During this time, the weight of the controls also decreased. This appears to be a definite response to a change in environmental conditions.

The changes in weight which followed the return of the animals to their original environment are of uncertain significance. There was, however, an immediate increase in the weight of the light animals and a decrease in that of the dark group, but the changes that occurred were probably complicated by other factors, or by the persistence of effects of previous exposures to one or the other of the two environmental conditions.

In this connection, it is important to note that the response to the first change of light environment was not immediate. The gradual increase in weight shown by the animals transferred from darkness to light may have been a reaction to the change in the mode of living, and this possibility cannot be disregarded merely because the controls showed a similar reaction at the same time. Still, the first definite

evidence of a response was shown by the light animals in the form of a rather abrupt increase in weight followed by a prolonged decrease. This came 5 or 6 weeks after these animals had been placed in the dark. It was 2 months, however, after the dark animals had been placed in the light before the increase in weight of this group assumed a form that was clearly indicative of a reaction to the new environment.

A delay in the reaction to light or to the exclusion of light did not occur at the beginning of the experiments, when the animals were first placed in the light or dark, which suggests that the effect produced by these fixed environmental conditions involves more than a mere gain or loss in weight. The manner in which the metabolic activities of the animal are affected appears to be just as characteristic as the extent of the effect produced. Referring first to the curves in Text-figs. 1 to 4 which show the changes in weights of animals living in the open laboratory (control conditions), it will be seen that there is nothing constant or characteristic in the form of these curves. In some instances there is an immediate and progressive increase in weight continuing over a period of months with eventual stabilization and perhaps a further increase at a later date. This type of curve is shown by the animals of Group III (Text-fig. 3). In other instances, the gain in weight is delayed or there may be an initial loss or a very slow increase. Moreover, it will be seen that at certain times, as in midwinter, the increase in weight is greater than at others, or a maximum is attained while all animals are apt to lose weight at other times (spring and early fall). Unfortunately, no systematic observations are available for the summer months.

These peculiar fluctuations in weight are, of course, not confined to control animals, but they are most clearly defined among animals living in the open laboratory, and the loss of weight that occurs during the spring and fall is clearly associated with moulting. If the weight curves of control animals have a characteristic feature, it is the occurrence of these periodic increases and decreases in weight, and it will be noted, that animals brought into the laboratory at or near the same time show a closer agreement with respect to the occurrence of these fluctuations than animals that are brought in at different periods of the year. This will be seen by comparing the control curves in Text-

figs. 1 and 2 with those in Text-fig. 4; the curve in Text-fig. 3 is exceptional.

The weight curves of animals exposed to neon light or living in an environment from which light is excluded are distinctly different from those of control animals. In the first place, animals placed under either of these conditions showed an immediate and decided increase in weight; the initial rate of increase was much the same in the two cases, if allowance is made for possible differences in the composition of groups. In the case of light animals, the gain in weight was continuous and gradually developed into a series of step-like increases marked by brief periods of stabilization or reduction in the rate of gain which were succeeded in turn by more rapid increases. A definite decrease in weight was extremely rare, and so far as we have been able to determine, occurred only with some irregularity in feeding, as during or immediately following a holiday period, or in connection with moulting or the development of disease.

The weight curves of animals living in the dark are equally characteristic. One of the most distinctive features is the occurrence of a definite loss of weight following the gain of the 1st month or 6 weeks. This gain followed by a loss forms the characteristic pattern of the curve which is repeated over and over again with little or no material change in level. The general level is usually that of the control animals, but it will be seen that the fluctuations do not necessarily coincide with those of any other group of animals.

We have emphasized the distinctive features of the weight curves because it has been found that in certain instances variations in the chemical composition of the blood of animals living under these conditions give evidence of the occurrence of movements of a similar nature. It is not our intention to discuss this aspect of the influence of light environment at this time, but in order to indicate something of the nature of the relation that appears to exist, the results obtained for blood calcium on two groups of rabbits have been plotted against those for body weight (Text-figs. 5 and 6). The actual values for body weight and for blood calcium have been smoothed in the same manner, so that the curves are strictly comparable. The form agreement between these curves is so close that there can be no doubt that in these particular instances the variations in body weight and blood

calcium are related.   It is evident, therefore, that conditions of living which affect one of these values may produce corresponding effects on the other in that a variation in one value may be reflected by the other.   This is as far as this phase of the subject will be carried at present.

Others have found that confinement of animals in the dark for varying periods of time and irradiation by such means as the carbon arc or quartz mercury lamp will affect the rate of growth, or gain in weight, as well as the chemical composition of tissues and body fluids (3), but comparatively little attention has been given to fixed environmental conditions, or to a study of effects produced by prolonged exposure to light of long wave-length.   It has been shown, however, that under appropriate conditions both the physical constitution or organic equilibrium of animals and the reaction to disease may be greatly affected by light of comparatively long wave-length such as that produced by Mazda lamps and the low pressure mercury arc in crown glass (4).

From the results obtained in these experiments, it is also evident that prolonged exposure to fixed environmental conditions, such as those represented by neon light or complete exclusion of light, may produce a profound effect on the animal organism (rabbit).   In the present instance, the influence of environmental conditions was shown by a marked effect on the ultimate weight attained and also on the rate of gain and the form of the weight curve.

The increase in weight that occurs with animals of the ages used is attributable, in part to skeletal growth, in part to muscular development, and in part to an accumulation of fat.   In general, the skeletal and muscular development, or growth in stature of animals exposed to neon light was greater than that of animals living in the dark; fat was also more abundant, but with few exceptions, animals living in the dark appeared to be well nourished and showed large accumulations of fat in all depots, even though the gain in weight had virtually ceased. In the one case, the effect produced suggests a definite promotion of growth with heightened metabolic activity while in the other, metabolic activity was apparently depressed or held in abeyance.   Control animals differed from those living in a fixed environment in that they

tended to show prolonged periods during which one or the other of these conditions prevailed.

When animals were placed in the light or dark for the first time, the response was comparatively prompt, but with continued existence in the fixed environment, it appears that a persistent, or almost fixed, tendency was established so that the effect produced on body weight by a subsequent change from one environment to the other was greatly delayed. At first sight, it might-appear that in this respect the effects of prolonged exposure to a fixed artificial environment differ from those of exposure to a natural light environment, but the difference may be more apparent than real as there is some evidence to indicate that tendencies established or accommodation to conditions that prevail during one period of the year, as in summer or winter, may persist for some time thereafter and modify the effects produced by a subsequent change in environmental conditions.

These results are of interest from still another point of view, namely, the denudation of body surfaces as a necessary means of obtaining effects from exposure to light. The effects produced may conceivably be increased by direct exposure of a skin surface, but these experiments show conclusively, as have others from these laboratories, that as a routine procedure, denudation is not necessary and in certain instances is undesirable as extensive denudation of body surfaces may give rise to conditions which will complicate the results of experiments.

In the present state of knowledge concerning the biological action of light, it is impossible to give a satisfactory explanation of the results obtained in these experiments. The present tendency is to correlate biological effects with the energy developed by rays of a given wavelength. From experiments carried out on this basis, it would appear that biological effects are confined very largely to rays in the ultraviolet region of the spectrum, or to rays with a wave-length of 3300 Ångström units or less. We wish to emphasize the fact, however, that there is a fundamental difference between a brief exposure of a living organism to light of given wave-lengths and prolonged existence in an environment of the same light; moreover, it is evident that results obtained under one set of conditions have no necessary bearing on the results that may be obtained under the other conditions. At-

tention may also be directed to the fact that results obtained by intense irradiation of experimental animals are usually complicated by a reaction to injury which makes it difficult to distinguish between that which represents a physiological response to radiant energy and that which represents a pathological reaction to the damage produced.

Our experiments deal with effects of light environment rather than irradiation. The results are clearly defined and are not complicated by a reaction to injury. For present purposes, it is immaterial whether the promotion of growth and nutrition that was observed among animals living in an environment of neon light is regarded as an effect of the small amount of short wave-length radiation or the larger amount of light of long wave-length. In either case, the illumination and the total energy involved are so small that they afford no basis for a satisfactory explanation of the effects produced. In all probability, the animals living under control conditions received a larger amount of light energy with higher illumination values than those exposed to the neon light, but in one case, the light environment was inconstant and subject to wide variation, while in the other it was virtually fixed. We regard this as an important factor. At any rate, it has been shown that, under certain conditions, differences in light environment which represent small amounts of light of comparatively long wave-length and small equivalents of energy are capable of producing clearly defined biological effects, and that the effects produced vary with the color or breed of the animal and are probably modified by still other factors, such as the age of the animal and the time at which the experiments are carried out.

SUMMARY.

The influence of light environment on the growth and nutrition of normal rabbits was studied by comparing the weight curves of animals living under different environmental conditions for periods of 4 to 8 months and the effects of change from one environment to another. Prolonged exposure to neon light was compared with confinement in the dark and exposure to diffuse, filtered sunlight of varying intensity.

The results of the experiments showed that growth and nutrition were greatly affected by the light environment in which the animals lived. The effects produced by a given environment varied with the

color or breed of the animal and appeared to be out of proportion to the differences in the intensity of the light or the energy represented.

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Neon Light

Fig. 1.

INFLUENCE OF LIGHT ENVIRONMENT ON THE GROWTH OF HAIR IN NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

BY WADE H. BROWN, M.D.

WITH THE COLLABORATION OF MARION HOWARD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In a previous paper,[1] it was shown that continuous exposure to neon light, or living in an environment from which all light was excluded produced definite and characteristic effects on the growth and nutrition of normal rabbits when compared with control animals living in the laboratory and receiving diffuse, filtered sunlight.

The influence of the 3 types of light environment on these animals was tested in still other ways. Parallel observations were made on the calcium and inorganic phosphorus content of the blood, and in the course of these investigations, it was noted that when the ears were shaved for bleeding, there was a striking difference in the growth of hair from one bleeding to the next. This difference in the growth of hair appeared to offer a satisfactory basis for a simple test of functional activity in the three groups of animals. At the time these experiments were in progress, Stevens was conducting a series of experiments in another laboratory of this Institute on the action of ultra-violet light and other agents on the growth of hair in rabbits, and the results obtained by him encouraged us to use the growth of hair over shaved areas as an index of cellular activity. The purpose of this paper is to report the results of the tests made.

In presenting these results, we wish to make it clear that it is not the purpose of this paper to discuss effects of light or of any other agent on the growth of hair as a problem in itself but merely to present the results of a series of experiments in which the growth of hair was used as an index of the proliferative activity of a particular group of cells.

[1] Brown, W. H., *J. Exp. Med.*, 1928, xlviii, 31.

Material and Methods.

The animals used for the experiments to be reported were the same as those used for the study of the influence of light on growth and nutrition,[1] so that a detailed statement of the general condition of the experiments need not be repeated.

The procedure employed in testing the proliferative activity of follicular cells was to compare the rate and extent of the growth of hair over a shaved area of the skin of rabbits that had been living in a given light environment for a long period of time. The conditions compared were continuous exposure to neon light, complete darkness, and a variable environment of diffuse, filtered sunlight. The tests were made as follows: the hair on one side was clipped and shaved over an area measuring approximately 8×5 cm. Care was taken to avoid trauma of any kind and to maintain uniformity in the location of the areas shaved and in the shaving itself.

The animals were examined at brief intervals throughout the experiments, first, for evidence of trauma, or of a traumatic reaction, and then for the purpose of determining the time at which growth of hair could be detected and the rate and extent of the growth that occurred until restoration was complete. The observations made on the growth of hair on the trunk were supplemented by observations of the same kind on one ear. In this case, a narrow band was shaved from the base to the tip of the ear along the course of the central vessels.

Three groups of 15 rabbits each were used for the tests. In each case there were 5 animals exposed to neon light, 5 in the dark, and 5 served as controls. Group I was composed of white rabbits, Group II of black, and Group III of gray, brown, and black animals. One experiment was carried out in midwinter when, normally, the proliferative activity of hair follicles is comparatively slight, and another at the time of the spring moult.

The animals of Group III were used for the first experiment. They were shaved December 29, 1926. At that time, they had been living in their respective environments for approximately 3 months. Observations were made under existing environmental conditions for 6 weeks. The light and dark groups were then interchanged and observations continued for 12 weeks after which the animals were restored to their original environments for an additional period of 4 weeks, giving a total observation period of 23 weeks (December 29, 1926, to June 8, 1927).

The animals of Groups I and II were used for the second experiment. They were shaved April 8, 1927 which was approximately $5\frac{1}{2}$ months after they were placed in their experimental quarters (October 22, 1926, to April 8, 1927). The records of hair growth cover a period of 40 days.

RESULTS.

The results obtained for the growth of hair on the side are presented graphically in Figs. 1 and 2.

The rectangular areas in these charts represent the shaved area; the proliferation of follicles and growth of hair are shown by the shading. A clear rectangle means

no reaction; line shading in one direction indicates proliferation of hair follicles with a short or thin growth of hair; cross-hatching a fairly long or thick growth of hair, while solid black indicates complete restoration over the area thus marked.

The results obtained for the growth of hair on the ears are not presented as they were essentially the same as those for the trunk. The only difference noted was a somewhat more rapid growth of hair on the ears which may have been influenced to some extent by the occasional use of xylol in bleeding or by trauma. Neither of these factors affected the results for the trunk.

Fig. 1.

Fig. 2.

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DISCUSSION AND CONCLUSIONS.

The difference in the response shown by animals living under different environmental conditions is very striking (Figs. 1 and 2). Within 5 to 10 days, 9 of the 15 rabbits that received neon light showed active proliferation of hair follicles with a definite outgrowth of hair, while among those in the dark there were none, and there were only 2 of the controls that gave any evidence of a reaction, and one of these (No. 5, Fig. 1) was not a true reaction. The narrow line of proliferating follicles seen in this animal was present at the time of shaving and did not increase thereafter; on the contrary, it diminished, indicating that the condition was merely an irregularity in the growth of hair such as is frequently seen among rabbits living in the laboratory. Differences of the order of those displayed by the three groups of animals during the first 2 weeks were maintained until the end of the experiments.

Comparing the results for the 2 experiments, it will be seen that among the animals living in an environment of neon light, the reaction was not quite as prompt in the first experiment (midwinter) as in the second (spring moult). In the second experiment, all animals showed an active growth of hair within 2 weeks, while in the first experiment, 2 of the 5 animals showed no reaction at a corresponding time and in one of these the growth of hair did not begin until towards the end of the 4th week. The end result was, however, essentially the same, but at the end of 6 weeks, restoration was more nearly complete in the first than in the second experiment.

Among control animals and those living in the dark, the differences between the 2 experiments were greater. None of the 15 control animals showed complete restoration of hair over the shaved area. In the first experiment, there was a fairly good growth of hair in 1 animal, but at the end of 23 weeks, this was still irregularly distributed. In the second experiment, the reaction was much more prompt and at the end of 3 weeks, 7 of the 9 animals showed a definite reaction as compared with none in the first, while at the end of 6 weeks, irregular patches of long hair were present in 4 of the 9 rabbits of the second experiment and none of the first.

A comparison of the results obtained for animals in the dark must take account of the fact that in the first experiment these animals

were transferred from darkness to an environment of neon light 6 weeks after they were shaved and remained in this environment for 12 weeks. The reverse change of environmental conditions did not affect the results for the light group, as the restoration of hair was virtually complete when the transfer was made. In the case of the dark group, however, there was a definite growth of hair in only one animal at the end of the first 6 weeks (a black rabbit); the skin of the others was as smooth and devoid of any evidence of reaction as though the hair had just been removed by some depilatory reagent. This condition was not changed in the slightest degree for 6 or 7 weeks. Scattered patches of proliferating follicles then appeared and gradually the hair began to grow; growth was not appreciably disturbed when the animals were again returned to the dark, so that at the end of the experiment, all of them showed a good growth of hair over practically the entire shaved area, but only 1 of them a complete restoration.

This result is especially important in comparison with the controls which showed a more active growth of hair during the first half of the experiment, but far less during the second half. Furthermore, beginning at about the 16th week, or 10 weeks after they were placed in the light, the growth of hair in the dark group was almost equal to that of the light group during the first 6 weeks of the experiment. This is also significant as it shows that the initial inactivity of the hair follicles of these animals was slowly abolished and replaced by a highly active condition. It is also of interest to note that the time required for the development of this condition in the skin is in fairly close agreement with the results obtained for the establishment of a definite increase in the rate of gain in weight.[1]

The results for the second experiment on animals living in the dark agree with those for the first in showing an initial state of comparative inactivity. The reaction, was however, much more prompt in the second than in the first experiment, and by the end of 6 weeks there were only 4 of the 10 rabbits that showed no reaction, while there were 2 animals in which the restoration of hair was complete over the greater part of the shaved area. In this experiment, the proliferation of hair follicles and the growth of new hair among the animals in the dark was almost equal to that of the controls, but still far less than that of animals in the light.

From a comparison of all results for the first and second experiments, it is evident that, irrespective of environmental conditions, there was a distinct difference in the promptness and constancy of the reaction displayed in the two cases. The difference between the two light groups was least as these animals, with few exceptions, showed an almost immediate response; the difference was greater between the two groups of controls with respect to the time, the constancy, and extent of the reaction, and still greater in the case of animals living in the dark. It is evident, therefore, that while complete exclusion of light may depress the proliferative activity of the follicle cells to a certain extent and exposure to neon light may increase their activity, in neither case is the effect such as to prevent or completely obscure the normal tendency to the occurrence of periods of heightened activity as at the time of moulting.

A further point of interest concerns the behavior of white as compared with black animals. It is not certain that a clearly defined difference in the reaction of the two classes of animals can be established, but there are suggestive differences. In the first place, the records show that in the light group of the second experiment (Fig. 2) the black rabbits reacted more promptly than the whites. This was true also for control, and probably for dark animals. At the conclusion of the experiment, however, restoration of hair over the shaved area was further advanced in the white than the black rabbits of the light group, while among the controls the situation was reversed with no decided difference in the dark group.

These differences are apparently slight and might be disregarded were it not for the fact that they agree absolutely with the results obtained for increase in body weight, and hence suggest that the two groups of functional reactions have a common basis which, in all probability, is to be found in an effect produced by the light environment on metabolic processes. This conclusion gains additional support from the parallelism between the effects produced on the growth of hair and the gain in weight of animals that were transferred from a dark to a light environment, as noted above. It is not to be inferred, however, that gain in weight and growth of hair are regarded as processes that are necessarily related, for, as was pointed out in a previous paper, a loss in weight is not infrequent at the time of moulting.

Summarizing the results of the experiments, it may be said that it was found that rabbits that had been living in an environment of neon light for a period of 3 months or more showed active proliferation of hair follicles and virtually complete restoration of hair over shaved areas within 5 to 8 weeks, while among animals living in the dark for the same length of time, or among controls receiving diffuse, filtered sunlight, the reaction was greatly delayed and, in the end, was irregular and incomplete. The growth of hair was, however, more active in all classes of animals during the spring than during the winter months, and there appeared to be a difference in the behavior of white and black animals.

It was also found that the skin of animals that were totally inactive after a prolonged existence in the dark became active after exposure to neon light for a period of 8 to 10 weeks.

As the results of these simple tests of the proliferative activity of the cells of hair follicles agree in every respect with those obtained for increase in body weight of the same animals, it is reasonably certain that the two sets of results have the same significance. Taken together, they show conclusively that conditions of light environment may have a profound influence on the animal organism (rabbit) and it is highly probable that an influence of some kind is exerted, irrespective of the wave-length or energy equivalent of the light concerned.

SUMMARY.

In a series of experiments dealing with the influence of light environment on normal rabbits, the growth of hair over shaved areas was used as an index of functional activity. The conditions compared were exposure to neon light, complete exclusion of light, and exposure to diffuse, filtered sunlight of varying intensity.

It was found that prolonged existence under these conditions affected the proliferative activity of hair follicles in a manner and to an extent comparable with the effects produced by the same environmental conditions on the growth and nutrition of the animals themselves.

RELAPSE PHENOMENA OF SPIRONEMA RECURRENTIS.

By HENRY EDMUND MELENEY, M.D.

(From the Laboratory of Tropical Medicine, Department of Medicine, Peking Union Medical College, Peking, China.

Our present knowledge of the interaction of host and parasite in relapsing fever may be summarized as follows.

At the time of the crisis which terminates the attack of fever, there is rapid agglutination and destruction of the spirochetes with the subsequent formation of immune bodies in the blood (Sawtschenko and Melkich (1); Novy and Knapp (2)). These substances are specific for the strain of spirochetes which was present during the preceding attack, but have no influence on the spirochetes of the succeeding relapse. The spirochetes of the relapse give rise, in turn, to immune substances which are specific for them but not for the spirochetes of the first attack (Levaditi and Roché (3); Jansco (4)). Some workers have encountered, in successive relapses, several modifications of the original strain of spirochetes (Kudicke and Feldt (5)), while others have found only two alternating strains (Cunningham (6)). During epidemics of relapsing fever, some investigators have found a remarkable uniformity in the strain causing the first attack (Jansco (4)), while others have found several different strains (Toyoda (7)). Passage through its natural intermediate host, the tick, has also been shown to produce a change in the serological reactions of the spirochete (Kroo (8)). The new strains of spirochetes produced by relapses have been found by some workers to remain immunologically different from the spirochetes of the first attack through many animal passages (Levaditi and Roché (3); Cunningham (6)), while others have described a gradual reversion to the original strain (Kudicke and Feldt (5); Toyoda (11)). In mice which have apparently recovered from the infection, either naturally or by arsenical treatment, the brain and sometimes other organs are infectious for new mice for a period of several weeks (Buschke and Kroo, (9)), and spirochetes have actually been found in very small numbers in the blood stream of patients during the interval between attacks (Kudicke, Feldt and Collier, (10)).

Plan of Work.

The present study was undertaken for the purpose of studying further the changes in serological reactions produced during the

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relapses of *Spironema recurrentis*. The animals used were the gray squirrel (*Sciurotamias davidianus*[1]) and the striped chipmunk (*Eutamias asiaticus*), both of which are native to North China. Chipmunks were used only when squirrels were not available.   In both of these animals intraperitoneal inoculation of blood direct from a Chinese patient suffering from louse-borne relapsing fever produced a non-febrile attack lasting usually 3 to 5 days, in which huge numbers of

TABLE I.

Typical Course of Relapsing Fever in a Splenectomized Squirrel (Sq. B, Chart 1).
March 25. Splenectomy performed.
March 29. Inoculated intraperitoneally with 0.5 cc. whole blood from a positive squirrel.

	Dates inclusive	Duration in days	Result of examination of tail blood				
			1st day	2nd day	3rd day	4th day	5th day
		days					
First attack..............	Mar. 30–Apr. 3	5	++	++	++++	+++	+++
First interval..............	Apr. 4–11	8	Daily examination negative for 8 days				
Second attack (1st relapse)...	Apr. 12–14	3	+	+	+++		
Second interval.............	Apr. 15–20	6	Daily examination negative for 6 days				
Third attack (2nd relapse)...	Apr. 21–25	5	+	+	++	++	+++

The number of plus signs indicates relative intensity of blood infection.   + = one spirochete to ten or more fields.   ++++ = 20 or more spirochetes to a single oil immersion field.
The tail blood was examined daily for 30 days after the third attack and was always negative.

spirochetes were present in the animal's blood. A second attack, however, never occurred, and the strain at first employed died out after the sixth transfer.
The observation of Tournade (12) that splenectomy increased the susceptibility of the wild rat to infection with *S. recurrentis* suggested that this procedure might produce relapses in squirrels and chipmunks,

[1] In a previous paper (13) this squirrel was erroneously called *Sciurus vulgarus*.

and this was found to be the case.   The effect of splenectomy on the course of the disease has already been reported (13).   Not only a second attack (first relapse) but a third attack (second relapse) occurred in many of the animals.   Table I shows a typical course of infection in a splenectomized squirrel.   Kritschewski and Rubinstein (14) have recently shown that splenectomy markedly increases the mortality of infection with *S. recurrentis* in white mice.

In the present experiments the original strain of spirochetes was transferred from squirrel to squirrel at the height of the first attack. When relapses occurred with this strain the relapse spirochetes were likewise transferred to separate series of squirrels.   These squirrels,

TABLE II.

Duration of Existence of Strains Maintained by Transfer.

Strain No.	Duration	No. of transfers
	days	
I	132	38
II	106	29
III	98	26
IV*	75	20
IV†	32	8
Va††	54	16
Va§	39	10
Vb	50	14
VI	22	6

  * Isolated from Sq. C, Chart 1; † isolated from Sq. D, Chart 1; †† isolated from Sq. F, Chart 1; § isolated from Sq. E, Chart 1.

in turn, usually had relapses, and when this occurred the spirochetes appearing in the relapses were tested against the monovalent immune sera produced by previously isolated strains.   Whenever a strain of spirochetes was encountered which could not be agglutinated by any of these sera it was considered to be a new strain and was preserved by transfer into new squirrels.

By this method six strains of spirochetes were isolated, which retained their specific agglutinating characteristics during the period of our observations, extending from March 21, to July 30, 1927.   Table II shows the duration of existence of the six strains.   The history of these strains is given in detail below.

Technique.

Splenectomy.—This was performed under ether anesthesia through a small transverse incision below the left costal margin. The pedicle was ligated, and the wound closed with silk sutures and metal clips. A localized area of necrosis frequently developed about the tail of the pancreas where this had been accidentally ligated. Very few animals died as a direct result of the operation, and general peritonitis did not occur.

Transfer of Spirochetal Blood.—Infection of the first squirrels was accomplished by the intraperitoneal injection of venous blood from a human case of relapsing fever. For transfer from squirrel to squirrel, blood was taken from the heart or the saphenous vein. 0.5 cc. of blood from an animal with a moderately heavy infection usually produced demonstrable blood infection within 12 to 18 hours.

Examination of Blood for S. recurrentis.—The tail blood of all infected animals was examined daily by dark-ground illumination. No animal was declared negative without a search of at least 5 minutes.
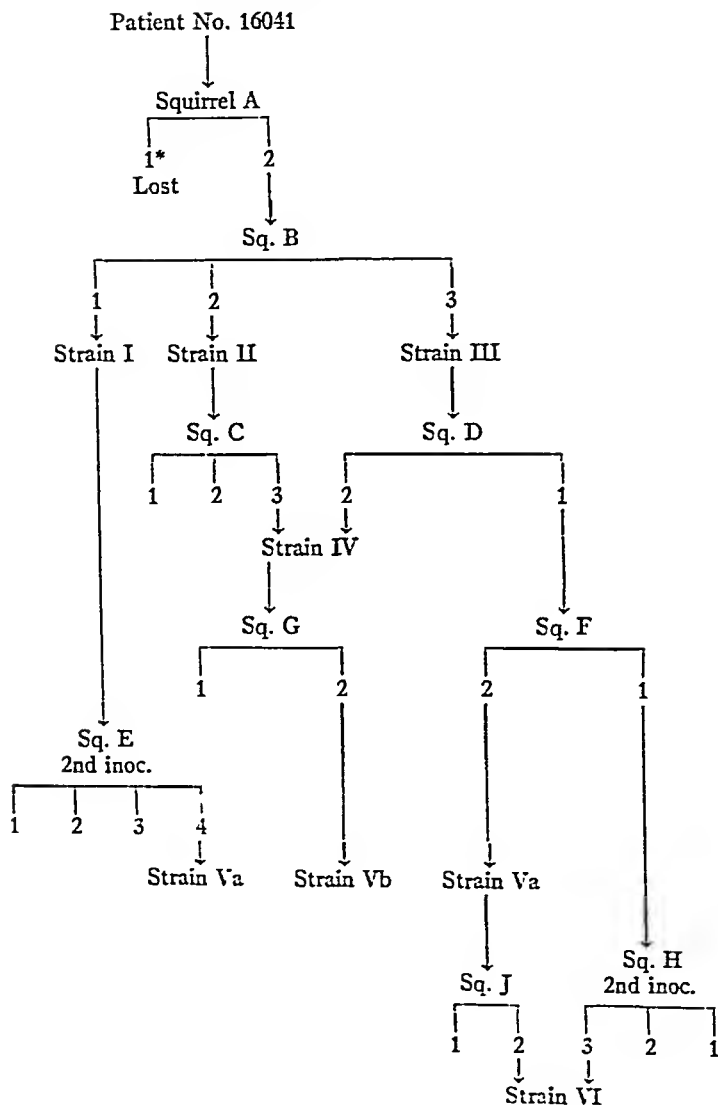
Collection of Immune Sera.—Blood was taken from the saphenous vein or by heart puncture. The sera were pipetted off from the clot and stored in the refrigerator. Sera appeared to retain their original agglutinative titer for over 2 months, that is, until the present study was completed.

Agglutination Tests.—Preliminary tests suggested that a dilution of less than 1–50 sometimes produced non-specific agglutination. This dilution was the lowest used in differentiating strains of spirochetes, but in measuring the curve of agglutinins in single animals dilutions of 1–10 and 1–20 were also used. Sera were diluted with normal saline, and controls consisting of saline and negative sera were employed. For the spirochete suspension the tail blood of strongly positive squirrels was used. This was found to be more practical than the removal of blood into citrate solution and the subsequent removal of the red blood cells, because the latter procedure sometimes led to the rapid death or spontaneous agglutination of the spirochetes. With moderate or large numbers of spirochetes in the blood, the red blood cells did not interfere with the reading of the tests. Tests were performed with slide-cover slip preparations and observations were made at once by dark-ground illumination. A second observation after half an hour was found to be of no value. This technique is practically the same as that employed by Cunningham (6).

History of Strains Isolated.

The original strain of spirochetes used for these observations was obtained from a Chinese woman suffering from relapsing fever in her first attack of fever. 4 cc. of whole blood were injected intraperitoneally into a squirrel (Squirrel A, Chart 1). This squirrel's spleen had been removed 5 months previously. Spirochetes appeared in the

CHART 1.

Lineage of Strains Isolated.

Patient No. 16041

Squirrel A

1* — Lost   2

Sq. B

1 — Strain I   2 — Strain II   3 — Strain III

Sq. C   Sq. D

1   2   3   2   1

Strain IV

Sq. G   Sq. F

1   2   2   1

Sq. E
2nd inoc.

1   2   3   4

Strain Va   Strain Vb   Strain Va

Sq. J   Sq. H
2nd inoc.

1   2   3   2   1

Strain VI

* Arabic numerals refer to attacks.

tail blood the day following inoculation and the attack lasted 5 days. It happened that no new squirrels were available at this time, so that the spirochetes of this attack were lost. After a negative interval of 7 days, spirochetes again appeared in the tail blood. This second-attack (first-relapse) strain of spirochetes was thereafter maintained in squirrels by transfer during their first attack, and became Strain I in the following experiments.

Chart 1 shows the lineage of the strains isolated. It has been reduced to the simplest possible form by omitting all animals except those from which new strains were isolated. It will be seen that Strains II and III originated from the second and third attacks, respectively, of a squirrel (Sq. B) inoculated with Strain I; that Strain IV was obtained from two sources, namely from the third attack of a squirrel (Sq. C) inoculated with Strain II and from the second attack of one (Sq. D) inoculated with Strain III. Strain V is divided into two closely related substrains, Va and Vb. Strain Va was obtained both from a fourth attack[2] in a squirrel (Sq. E) inoculated with Strain I and from a second attack in one (Sq. F) inoculated with Strain III. Strain Vb appeared in the second attack of a squirrel (Sq. G) inoculated with Strain IV. At first all of the Strain V spirochetes appeared to be identical, since they were each agglutinated by the monovalent serum produced by the other. It was found, however, that the spirochetes designated as Strain Vb had a definite relationship with Strain III, while those designated as Strain Va failed to show this relationship but did show a relationship to Strain II (see later section on Relationship of Strains). Finally Strain VI originated from the third attack of a squirrel (Sq. H) inoculated with Strain III and from the second attack of a squirrel (Sq. J) inoculated with Strain Va. It was necessary to close the present study soon after the isolation of Strain VI, because of inability to secure more animals.

Sequence of Strains.

A consideration of the sequence in which the different strains of spirochetes appeared in relapses is important. The identity of each relapse strain was determined either by testing the spirochetes which

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[2] The last three of these attacks were produced by a second inoculation with Strain I.

appeared during an attack against monovalent immune sera, or by observing what additional agglutinins appeared in the animal's blood after the attack. In some cases both procedures were used. Tests of an animal's serum after an attack did not always indicate clearly what strain had been present during the attack, for agglutination was sometimes equally strong with the sera of two different strains. This is because of the relationship existing between certain strains, as will be shown later. Table III contains those cases in which there was a clear-cut indication of the sequence. It will be seen that in animals inoculated with Strain I, Strain II was the only strain which appeared in the second attack, and Strain III the only one which appeared in the third attack. When other strains were inoculated, however, the sequence was not so uniform. Strain II was usually followed in the

TABLE III.

Sequence of Strains in Relapses.

Strain inoculated	Number of cases in which each strain appeared in subsequent attacks (relapses)													
	Second attack strain							Third attack strain						
	I	II	III	IV	Va	Vb	VI	I	II	III	IV	Va	Vb	VI
I	0	9	0	0	0	0	0	0	0	4	0	0	0	0
II	1	0	7	0	0	0	0	0	0	0	2	1*		0
III	0	0	0	2	1	0	0	0	0	0	0	0	0	1
IV	0	1	2	0	0	3	0	0	0	0	0	0	0	1
Va	0	0	1	0	0	0	1							

* Immune serum of this squirrel agglutinated spirochetes of both Strains Va and Vb.

second attack by Strain III, but once there was a recurrence to Strain I, and in the third attack Strain IV appeared twice and Strain V once. Strain III was followed in the second attack by Strain IV or Va, showing similarity here to the third attack after inoculation with Strain II. Strain IV was followed in the second attack three times by Strain Vb, twice by Strain III and once by Strain II. Strain Va was followed once by Strain III and once by Strain VI. Strain VI appeared also in the third attack once after inoculation with Strain III and once after inoculation with Strain IV. In short, inoculations with Strain I gave a uniform sequence, while after inoculations with other strains

the sequence of strains was not uniform.   In some cases reversion to earlier strains occurred, while in other cases new strains appeared.

Although the figures presented in Table III are small for most of the strains, they are sufficient to illustrate an important principle connected with the development of relapses.   This principle is the relationship of strains, on the basis of which all except one of the sequences which are shown in Table III can be explained.   This relationship between certain strains will now be considered.

Relationship of Strains.

Cunningham (6), in his experiments with squirrels in Madras, India, was able to isolate only two strains of spirochetes, and these strains alternated in their appearance.   Whenever his Strain I was inoculated Strain II appeared in the relapse, and whenever Strain II was inoculated Strain I appeared in the relapse.   In the present work,

TABLE IV.

Agglutinins in Serum of Squirrels after First Attack.

Strain inoculated	No. of sera tested	No of sera containing agglutinins in 1-50 dilution for strains			
		I	II	III	IV
I	10	10	0	0	0
II	11	0	11	0	0
III	5	3	0	5	0
IV	7	0	2	0	7

although the strains already described were definitely distinct, certain relationships were found to exist which correspond in principle with Cunningham's findings.

These relationships were demonstrated by the agglutination tests performed with sera taken from animals after the completion of one or more attacks.   Table IV shows the results of agglutination tests performed with sera taken after the first attack of Strains I, II, III and IV.   It will be noted that, in the case of serum from Strains I and II, agglutinins were demonstrated only for the homologous strain of spirochetes.   In the case of Strain III, however, three out of five animals developed agglutinins not only for Strain III but also for

Strain I. Similarly in the case of Strain IV, two animals out of seven developed agglutinins for Strain II as well as for Strain IV. This indicates a relationship between Strains I and III and between Strains II and IV.

The relationship between Strains I and III is further demonstrated by studying the agglutinins developed after the second attack in squirrels inoculated with Strain II. The results of these tests are shown in Table V. It will be seen that nine of the ten sera tested still possessed the agglutinins for Strain II which they had acquired as a result of the first attack. As a result of the second attack, all ten of the sera contained agglutinins for Strain I and five of them possessed in addition agglutinins for Strain III. There was more uniformity of agglutinin formation for Strain I than for Strain III, although, as has been shown already in Table III, the Strain III

TABLE V.

Agglutinins in Serum of Strain II Squirrels after Second Attack.

No. of sera tested	No. of sera containing agglutinins in 1-50 dilution for strains			
	I	II	III	IV
10	10	9	5	0

spirochetes appeared more often than Strain I in the second attack of squirrels inoculated with Strain II.

Strain Va was found to be related to Strain II, by the fact that after a first attack with Strain Va the animal's serum in two cases out of three agglutinated both the Strain II and the Strain Va spirochetes.

A relationship was shown to exist between Strain III and Strain Vb, by the fact that the Strain III spirochetes were agglutinated by univalent Strain Vb serum in all of the seven cases where the test was performed. Strains III and Vb were not identical, however, since the Strain Vb spirochetes were never agglutinated by univalent Strain III serum.

Strain VI was isolated such a short time before the close of the study that its relationship to other strains could not be investigated by means of agglutination tests.

In recapitulation it may now be stated that relationships were shown by agglutination tests to exist between Strains I and III, between Strains II and IV, and II and Va, and between Strains III and Vb. If one refers again to Table III it is evident that these relationships are all between strains which occurred, or might theoretically occur, in alternate attacks.   If we now apply this observation to Strain VI in an attempt to ascertain its relationship to other strains, it appears (Table III) that this strain was related both to Strain III and to Strain IV, since it was responsible for the third or alternate attack in animals inoculated with each of these strains.   Strains which were found to be related to one another did not occur in consecutive attacks, except once, when Strain II spirochetes appeared in a first relapse of Strain IV.

Reinoculation Experiments in Non-Splenectomized Squirrels.

Twenty-four squirrels were inoculated without removing the spleen and had no relapse.   After an interval of 10 to 20 days, each one was reinoculated with the strain with which it had been infected originally. All except one showed itself immune, the tail blood remaining negative after the reinoculation.   Following this, other strains were inoculated in succession, with shorter intervals between inoculation.   The inoculations were usually successful, producing a blood infection lasting from 1 to 5 days.   No squirrel, however, became infected with more than four strains.   Among the twenty-four squirrels in this series, the results were as follows: 6 squirrels became positive to 4 different strains, 11 squirrels became positive to 3 different strains, 6 squirrels became positive to 2 different strains, and 1 squirrel became positive to 1 strain only.

The successful inoculation of as many as four different strains in succession emphasizes the individuality of these strains.   The reinoculations were made at such short intervals that loss of immunity cannot be held responsible for the successful reinfections, for it is usual for antibodies against a given strain to be demonstrable in an animal's blood for at least a month after an attack with that strain.   The following protocol illustrates this type of experiment.

Protocol.—Squirrel 1. Spleen not removed. Inoculated with Strain I. Tail blood positive for 3 days. After 16 days negative the squirrel was reinoculated with Strain I. Tail blood negative for 4 days. Then it was inoculated with

Strain II.   Tail blood positive for 2 days.   After 9 days negative, it was reinoculated with Strain III.   Tail blood positive for 2 days.   After 5 days negative, it was reinoculated with Strain IV.   Tail blood positive for 3 days.   Total interval from second inoculation with Strain I to inoculation with Strain IV, 22 days.

The agglutinins and protective immune substances which were developed by a single attack seemed to be about equal in strength in normal and in splenectomized squirrels.   Reinfection with a previously inoculated strain was successful only once in a normal and once in a splenectomized animal.   Agglutinins to a titer of 1–12,800 frequently developed in the serum of both normal and splenectomized animals. The strength of the agglutinins seemed, in general, to be directly proportional to the intensity and duration of the attack.   It may be stated that neither specific immunity nor agglutinin formation seemed to be influenced by the presence or absence of the spleen.

DISCUSSION.

A. Strain Relationships, Relapses and Cure.

The fact that the strains of spirochetes which appeared spontaneously in consecutive attacks in splenectomized squirrels did not (with one exception) appear to be serologically related to each other, while those which appeared in alternate attacks were so related, is explainable on the basis that, at the close of an attack, the spirochetes find themselves in an exceedingly unfavorable environment, in which only those can survive which possess, or can quickly develop, biological affinities most different from those of the original strain.   These biologically different spirochetes produce the second attack, at the close of which they themselves encounter a very unfavorable environment.   Again only those can survive which possess, or can quickly develop, biological affinities which are very different from the second strain.   Since the unfavorable conditions which were present at the close of the first attack probably still exist at the end of the second attack, the original strain cannot again appear, but a third strain of spirochetes does appear, whose biological affinities are more like those of the original strain than like those of the first-relapse strain.   Assuming that a few organisms survive in the brain and blood stream even after the last attack, a fact which Buschke and Kroo (9) and Kudicke, Feldt and

Collier (10) have established, it is probable that clinical cure occurs when the surviving spirochetes find their environment too unfavorable for further multiplication in any great number.

The question arises whether the spirochetes which survive after an attack already possess biological affinities different from the original strain before they meet the unfavorable conditions which cause the destruction of their fellows, or whether their biological affinities change as a result of the unfavorable environment. The evidence at hand seems to indicate that the change does not occur before the development of the unfavorable environment but is rather the result of it; for if spirochetes which were biologically different from the original strain had existed before the crisis, one would expect them to have multiplied parallel with the original strain and to have given rise to antibodies in demonstrable amount, which they did not do.

The number of attacks which will occur before the medium becomes too unfavorable for the further occurrence of relapses must depend upon the interplay of several biological factors. Among these the three following are important:

(1) *Differences between Types of Spirochetes.*—It is well known that in the tick-borne type of human relapsing fever, the attacks are more numerous, are of shorter duration and occur at shorter intervals than in the louse-borne disease. The shorter attacks are probably due to a biological difference in the parasites, while the shorter intervals and larger number of attacks may be due, in part at least, to a less intense stimulation of immune bodies in the host as a result of the shorter attacks.

(2) *Susceptibility of Host.*—Certain animals like the guinea pig are naturally refractory to infection with *S. recurrentis.* Others, like the species of squirrel used in these experiments, normally have one attack but no relapse. Man is the ideal host and usually has the largest number of relapses.

(3) *The spleen* apparently plays an important rôle in preventing a multiplication of the parasites in animals like the wild rat which are naturally refractive to infection (Tournade (12)). It also prevents relapses in moderately susceptible animals like the Chinese squirrel. The exact nature of this protection is at present unknown, but the above observations indicate that it is not of the same nature as the

specific immune bodies which are produced in response to the infection. Two facts support this conclusion. In the first place, non-splenectomized animals could usually be reinfected by the inoculation of a second strain of spirochetes, although a spontaneous relapse never occurred in them. This indicates that the spleen did not produce any wider range of immune bodies in normal animals than in splenectomized animals. It also indicates that the protective power of the spleen is relatively slight, since a second attack which would not occur spontaneously could be produced by massive reinoculation. In the second place, splenectomized animals developed as efficient an immunity and as strong agglutinins against the organisms which appeared in their blood as did non-splenectomized animals. This indicates that the protection afforded by the spleen is not due to its production of specific antibodies. Further light on the protective function of the spleen might be obtained by determining whether, at the close of the first attack in animals which do not have a relapse, the spirochetes are all killed; or whether a few survive as they do in animals which have relapses. If they are all killed, the spleen would seem to assist in their destruction, whereas if they persist over a considerable period of time, it would appear that the spleen merely has the power to inhibit their multiplication.

B. Zones of Antibody Formation.

It will be recalled that when Strain III, IV or V was inoculated into an animal, serum taken after the first attack often agglutinated not only the strain of spirochetes inoculated, but also a related strain. When Strain I or II was inoculated, however, serum taken after the first attack agglutinated only the strain inoculated. To understand this, one must remember that the designation of any strain of organisms as Strain I is entirely arbitrary, and that it simply means that one does not possess any preceding strain. Our Strain I was a first relapse spirochete from a squirrel which had been inoculated from a patient. We know that a change of strain must have taken place in the squirrel. Another change may have taken place in the louse which transmitted the disease to the patient, and it is possible, also, that the louse acquired its spirochetes from another human being during a relapse. If we had possessed all of these preceding strains of spiro-

chetes, it is reasonable to suppose that some of them could have been agglutinated by sera taken after the first attack with our Strain I or Strain II.   There is no reason to suppose that Strains I and II were not capable of giving rise to as wide a range of antibodies as the other strains which were isolated in these experiments.   This fact is emphasized because it is important in considering the serological reactions in any group of spirochetes, and has apparently been overlooked in much of the preceding work on relapsing fever.

There is another conception, however, which may explain why our Strain I spirochete was sometimes agglutinated by monovalent Strain III serum, whereas our Strain III spirochete was never agglutinated by monovalent Strain I serum.   When Strain III originally developed in the third attack of an animal inoculated with Strain I, it developed in the presence of antibodies which would destroy any Strain I spirochetes which might appear.   However, when these Strain III spirochetes were inoculated into a new animal which possessed no antibodies against Strain I, spirochetes of the closely related Strain I which might develop during the first attack would not be destroyed, but would multiply along with the Strain III spirochetes, and give rise, at the end of the attack, to Strain I antibodies in serum which was supposedly monovalent for Strain III.   If this conception is correct, one must assume that, since Strain I spirochetes were agglutinated by some Strain III sera but not by others, the Strain I spirochetes developed in some animals inoculated with Strain III but not in others.

Kudicke and Feldt (5) found that a component of their Strain I (Ausgangsstamm) developed in their relapse strains when these were passed through normal mice, and that this component increased in subsequent passages until the relapse strains ultimately reverted completely to their Strain I.   In the present study, no reversion to an older strain was observed.   The development of agglutinins for older, closely related strains was the only approach to such a reversion. Each strain possessed the same agglutination reactions at the end of our study as it had after its first transfer to a new squirrel.   This coincides with the observations of Cunningham (6), who also worked with squirrels.   Toyoda (11), on the other hand, who worked with mice, found that his relapse strain reverted to the original strain after

eighteen passages. Whether the type of experimental animal had anything to do with this difference in results is a question which deserves further investigation.

It seems probable that each strain of spirochetes which appears during an attack of relapsing fever contains its own peculiar group of components, the exact range of which depends largely upon the range of the antibodies which were present in the environment in which the strain originated. When a number of relapse strains are isolated in experimental animals by artificial transfer, it is apparent that the components making up those strains may overlap to a considerable degree.

Fig. 1 has been constructed in order to illustrate the relationships of our strains and the gradual increase in the complexity of their sero-
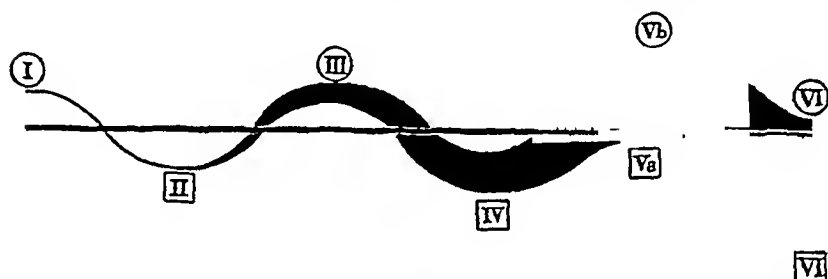
FIG. 1. The broadening zone of strain relationships in the descendants of Strain I.

logical relations. The wavy form of the curve represents the alternation in serological relationships between the spirochetes which appeared in consecutive attacks. The strains above the base line and enclosed in circles were related to each other, and those below the base line and enclosed in squares were likewise related. The curve is linear as far as Strain II because the sera produced by Strains I and II failed to agglutinate any other strains. The gradual broadening of the curve beginning just beyond Strain II, until it ultimately extends both above and below the base line at Strain V, represents the gradually broadening zone of relationship among the strains which were descendants of Strain I. The curve must be broader at III than at I because Strain III sometimes included a Strain I component, but was not identical

with Strain I.   At V the curve must extend both above and below the base line, because Strain Vb was related to Strain III, while Strain Va was related to Strain II.   At VI the curve must also extend both above and below the base line, because, since Strain VI appeared as the third-attack spirochete both in an animal inoculated with Strain III and in one inoculated with Strain IV, it was apparently related to both of these strains.

It is probable that a similar curve could have been constructed if the work had been begun either with an earlier strain than Strain I or with one of the later strains, say Strain V.   In the former case the curve would show broader relationships in the region of the present Strain I, perhaps carrying it both above and below the base line.   In the latter case, on the other hand, the curve would be linear for the distance from Strain V to Strain VI where it is now broadest.

There is evidence that a number of strains of *S. recurrentis* are present among human cases in an endemic region like North China. While the present work was in progress two other human cases yielded strains of spirochetes which could not be agglutinated by any of our sera.   The components making up these strains evidently did not include any of those which made up our strains.   Toyoda (7) also found that among ten cases of relapsing fever in a Manchurian mine there were at least three different strains of spirochetes.   How many different immunological strains of *S. recurrentis* there may be in China, or in the entire territory where the louse-borne relapsing fever is found, could be determined only by the collection and testing of organisms from a very large number of cases from various regions.

The serological studies of many previous workers, as well as the present experiments, have demonstrated that agglutination and immunity tests are not satisfactory criteria for differentiating the spirochetes of relapsing fever from various parts of the world.   Until some better criterion for their differentiation appears, it is reasonable to consider that all the spirochetes of relapsing fever belong to one species, *S. recurrentis*.   Within this species there are two biological varieties, the louse-borne parasite, which everywhere produces relatively long attacks separated by long intervals in human beings, and which is difficult to transfer to white rats and mice; and the tick-borne parasite, which produces short attacks separated by short intervals in human

beings, and which is easily transferred to white rats and mice. The erection of several species, because of geographical or immunological differences, or differences between vectors, seems to be unwarranted.

SUMMARY AND CONCLUSIONS.

1. Squirrels and chipmunks were found to be susceptible to infection with the *Spironema recurrentis* of North China, but no relapses occurred in normal animals.

2. Splenectomy caused an increase in the intensity of the infection and the appearance of one or two relapses.

3. By inoculating splenectomized squirrels with a single human strain of spirochetes, six different strains were produced as a result of relapses.

4. The strains all retained their specific agglutinating characteristics during the period of observation. The oldest strain was observed for 132 days and through 38 transfers, the strain last isolated for 22 days and through 6 transfers.

5. The sequence of strains in relapses was not always the same. Sometimes new strains were produced, at other times there was reversion to an older strain.

6. Certain relationships between strains were established. The related strains were always those which appeared, or theoretically might have appeared, in alternate attacks. In only one case did related strains appear in two consecutive attacks.

7. Squirrels whose spleens were not removed had only one attack and developed immune substances only against the strain inoculated or against closely related strains. It was possible by reinoculation to produce infection in such squirrels with as many as four different strains in succession.

8. The spleen has a protective influence against the development of the relapse in the squirrel, but apparently does not control the formation of specific immune substances.

9. An explanation of the clinical course of relapsing fever is suggested on the basis of the observations recorded.

10. There is, at present, no justification for the division of the spirochetes of relapsing fever into different species.

The writer is indebted to his colleagues, Doctors, C. U. Lee, Samuel Zia and E. G. Nauck, for assistance in various parts of this work, and also to his Chinese technicians, without whose conscientious cooperation the work could not have been performed.

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FACTORS INVOLVED IN THE PRODUCTION OF IMMUNITY WITH PNEUMOCOCCUS VACCINE.

I. ACTIVE AND PASSIVE IMMUNITY DURING THE FIRST SEVEN DAYS AFTER INJECTION OF ANTIGEN.

By ALVAN L. BARACH, M.D.

WITH THE TECHNICAL ASSISTANCE OF MAX SOROKA.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

Inasmuch as pneumonia is a self-limited disease ending by crisis or lysis generally between the 7th and 10th days, 3 to 5 days are frequently afforded in which an attempt may be made to produce active immunity before the natural termination of the disease takes place. At the suggestion of Dr. A. R. Dochez, a study was therefore undertaken of the onset and rate of development of pneumococcus immunity. As a result of recent work in oxygen therapy, it appeared to the author (1) that the life of the pneumonia patient was at times prolonged by the inhalation of 40 to 50 per cent oxygen, a circumstance that would further increase the possibilities of pneumococcus vaccine in an individual case. Any procedure that would initiate an earlier activity of the immunity mechanism seems therefore to have therapeutic possibilities. We wish to report preliminary animal experiments that have a bearing on this problem.

Historical.

In reviewing the subject, we shall present only such work as is concerned with the demonstration of specific immunization to the pneumococcus, and shall therefore omit the clinical reports of pneumococcus vaccine in the treatment of pneumonia as being apart from the present purpose. The development of immunity after introduction of pneumococcus organism has been shown by a number of workers. A great variety of methods of preparation of the organism has been used, employing the intact cell and extracts or solutions of the cell. The onset of demonstrable immunity in the studies of different investigators has been generally

between the 5th and 14th day after injection. Deviations from these results will be taken up in the individual instances. As we are concerned particularly with the attempt to secure a vaccine that would be applicable to the treatment of pneumonia, we shall stress chiefly the development of immunity during the first 7 days after injection.

The first demonstration that the serum of animals injected with pneumococcus possessed immune properties was made in 1891 by Foa and Carbone (2), Emmerich and Fowitzky (3), and G. and F. Klemperer (4). Neufeld (5) (1902) employed the bacteria separated from broth by centrifugalization, believing that the substances formed in the medium during growth were harmful. Wadsworth (6) (1912) found that the immunity produced by whole cultures was greater than that obtained by the injection of the bacteria alone. He also employed filtrates from the culture material after precipitation with ammonium sulfate. In both instances active immunity was produced in the rabbit 8 to 13 days after the injection. In 1913 Dochez and Gillespie (7) published their classification of pneumococci which made possible more specific study of individual strains. Lister (8), employing a similar classification in South Africa, made an intensive study of prophylactic inoculation against pneumococcus infection in the rabbit and in man. He found the factor of dosage was of considerable importance; doses limited to hundreds of millions of pneumococci were of little value, whereas 1 to 10 billion organisms administered intravenously both in the rabbit and in man were effective in immunizing the subject.

After three intravenous injections at weekly intervals, the rabbit was able to withstand a lethal dose of virulent pneumococcus culture. 4 to 5 days after the first injection, the serum of the animal in a few instances showed evidence of agglutinins when 3 volumes of serum were used to 1 of culture. One African native who received an intravenous inoculation of 10 billion the first dose, 20 billion the second dose, and 40 billion the third dose, showed agglutinins (with 3 volumes of serum) 6 days subsequent to the first injection. The vaccine was prepared from 18 hour blood agar cultures.

When the vaccine was administered subcutaneously, using from 2 to 12 billion organisms, no agglutinins were demonstrated even after three injections. When 20 to 30 billion doses were administered, agglutinins appeared 7 days after the second injection. In a later article, Lister (9) took his culture from human blood agar slants, inoculating it into trypsinated bullock heart broth for 8 hours, and then transferring this culture into similarly prepared broth for an incubation period of 6 to 7 hours. The cocci were finally suspended in saline, heated to 60° for 1 hour, and phenol added to a concentration of 0.5 per cent. Other preparations of vaccine such as (1) the filtrate after passing broth cultures through Berkefeld filters, (2) the cocci separated from broth cultures by electrolytic methods, (3) the pneumococci from 40 hours incubation in glucose broth cultures he found relatively inert as immunizing agents.

Still other types of vaccine have been employed: sensitized bacteria (Levy and

Aoki (10)), bacteria subjected to freezing (Cole (11)), bile extracts of pneumo-coccus (Neufeld (12), Vetrano (13), Cole (11)), glycerol extracts (G. and F. Klemperer (4)), and cultures precipitated by alcohol and dissolved in water (Wadsworth (6)). In all these instances, the immunity was slight, according to Cole (11), and never as good as from the injection of heat-killed bacteria. He found that better immunity was established in the rabbit by small daily doses than by larger doses at weeky intervals, agglutination (+ + with equal parts of serum and culture) appearing on the 11th day after the first injection. The serum also protected a mouse against 0.001 cc. of virulent pneumococcus culture.

Wadsworth (14) recently recorded quantitatively the degree of immunity which he obtained with a standardized vaccine made of meat infusion broth with 0.5 per cent glucose. After three subcutaneous injections of Type II vaccine at weekly intervals, there was slight active immunity after the last injection. Thus, of three mice injected with 0.00001 cc. culture, one died, and of three injected with 0.0001 cc. culture, two died.

Larson and Nelson (15) reported that the addition of sodium ricinoleate to a virulent culture of pneumococcus so that the final dilution of the soap is 0.1 per cent caused the organisms to lose their pathogenicity. When 10 cc. of such a culture were injected intravenously into a rabbit, large amounts of agglutinins were present in the blood stream 24 hours after. Cecil (16) has produced active immunity in monkeys after three subcutaneous injections of Pneumococcus Types I and II and to a less extent in Type III. After a single large subcutaneous injec-tion in man of 8 billion each of the three types, agglutination (+ in 1–1 dilution) was observed in some instances 7 days after. The vaccine was made by growing the organisms in 0.5 per cent glucose broth for 12 to 14 hours. Mackenzie (17) found that an intraperitoneal injection of 0.25 cc. of a heat-killed 18 hour culture produced active and passive immunity in guinea pigs 6 days after the injection although no agglutinins were demonstrable. Perlzweig (18) noted active im-munity 10 days after a single subcutaneous injection of Pneumococcus Type I in mice. He also immunized mice actively with the protein fraction obtained by treating pneumococci with anhydrous sodium sulfate and by solution of pneumo-cocci in bile salts and precipitation with alcohol. Ferry and Fisher (19) found that the centrifugate from broth cultures or saline suspensions of pneumococcus were more effective as an immunizing agent than the sedimented bacteria. White mice who were given two doses of 0.5 cc. of these extracts subcutaneously at weekly intervals were immune against 0.001 cc. of culture 1 week after the last injection. Wright (20) found that immunized rabbits cleared the blood of injected virulent pneumococci more effectively than normal rabbits. This increased resistance to septicemia was observed as early as the 3rd day following a single intravenous injection of heat-killed bacteria. Armstrong (21) has reported passive immunity in rabbits and active immunity in mice both beginning on the 4th day (occasionally on the 3rd day) and increasing to the 6th day after injection of killed pneumococcus vaccine.

In 1917 Dochez and Avery (22) pointed out that the pneumococcus elaborates a soluble specific substance which is found in the broth culture of the organism and frequently in the body fluids of the host as well. Recently, Avery and Heidelberger (23) have separated the constituents of the pneumococcus cell into two chemically and immunologically distinct substances, one protein and the other carbohydrate. The protein fraction is less specific than the intact bacterial cell, creating antibodies for the protein common to all groups of pneumococci when injected into animals. The carbohydrate, which has been shown to be of polysaccharide nature, is highly and specifically reactive only with the antibacterial serum of the same type of pneumococcus as that from which the substance is derived. However, when dissociated from combination with other cell elements, it is incapable of inducing antibody formation. They state that solutions and extracts of pneumococci behave as solutions of pneumococcus protein, *i.e.*, produce antibodies reactive only with the protein. Sera prepared from filtered solutions of disintegrated cells free from formed elements fail to exhibit any of the dominant type-specific properties which characterize sera obtained by immunization with whole bacteria. Schiemann and Casper (24) very recently reported that a specific soluble substance which they made employing a technique similar to Avery and Heidelberger was antigenic.

The literature recited above presents considerable divergence of opinion in regard to various problems in pneumococcus immunity. Our experiments have mainly emphasized two factors: (1) the onset and rate of development of pneumococcus immunity, (2) the character of immunity produced by the use of the intact organism as compared with that produced by a solution or extract from the bacterial cell.

Methods.

Numerous experiments have been conducted in the effort to prepare an antigen which would be particularly effective in producing early immunity. The detail of these findings will be considered at a later date. A fact originally mentioned by Neufeldt was suggested by our experience, namely, that the highly virulent organism provokes a more marked immunity than an organism of low virulence. We have attempted to prepare our antigen from a pneumococcus culture that was fatal to a mouse in 10–7 dilution, and preferably passed through a mouse immediately before using.

Human serum was employed in a concentration of 2.5 per cent in beef infusion broth in order to preserve virulence to the last stage and in order to augment

growth. The addition of glucose for stimulating growth did not appear to he satisfactory because of the readiness with which the organism was altered by growth in the culture medium. Young cultures were employed also with the idea of preventing autolysis. The incubation period was varied between 6 and 11 hours without appreciably influencing this factor.

Four different antigens were prepared in the following manner: A virulent Pneumococcus Type II (or Type I) culture was passed through a mouse in a preparation of the vaccine. 0.2 cc. of the heart's blood obtained from the animal immediately after death was inoculated into a test-tube containing 5 cc. of beef infusion broth with 2.5 per cent of human serum. After 8 hours incubation, this was used to inoculate flasks containing 250 cc. of similarly prepared broth, in the proportion of 0.1 cc. inoculum to 5 cc. hroth. Incubation was carried on for 8 to 11 hours as was desired. The culture was then centrifuged, the supernatant broth poured off and saved. Care was taken not to loosen the sedimented hacteria at the bottom of the centrifuge tube which was now carefully rinsed with distilled water to wash off all hroth adhering to it. Distilled water was finally added to dilute the bacteria to a concentration of 1 billion organisms to 1 cc. of water. (A nephelometer was used and checked hy the Wright counting method.) The vaccine was sterilized by exposure to 60° for 1 hour. After it had cooled, tricresol was added to a concentration of 0.3 per cent. This is called in the text the 8 or the 11 hour serum vaccine.

The supernatant broth saved from the original culture was passed through a Berkefeld candle, bottled, and placed in the ice box. The preservative was added after culture had demonstrated no organisms had passed through the filter. This is called the hroth filtrate.

A second lot of serum vaccine before sterilizing is shaken by hand for 5 minutes and centrifuged. The supernatant fluid is passed through a Berkefeld filter, bottled, and placed in the ice hox. If sterile on culture, the preservative is added. This is called the filtrate of the shaken hacteria. The sediment remaining from the above is taken up in distilled water, heated for 1 hour at 60°, and allowed to cool hefore the addition of tricresol. This is called the washed hacteria vaccine.

Experiments.

Experiment 1.—A series of white mice were injected intraperitoneally with three different antigens derived from Pneumococcus Type II: (1) 11 hour serum vaccine, (2) Berkefeld filtrate of shaken bacteria, (3) washed bacteria vaccine. Each antigen was injected into five mice daily for 5 days. The dose of the serum vaccine and the washed bacteria vaccine was 0.2 cc., representing 200 million organisms. The dose of the filtrate was 0.4 cc., derived from 400 million organisms. At the end of the 5th day, all mice received an intraperitoneal injection of Type II culture in varying dilutions, as shown in Table I. The test culture was fatal to a mouse in 10–7 dilution.

TABLE I.

Active Immunity in Mice after Intraperitoneal Injection of (1) 11 Hour Serum Vaccine, (2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria Vaccine.

Antigen used and dose	Day after vaccination	Survival after Pneumococcus II injection of culture			Remarks
		.0001	.00001	.000001	
Vaccine 0.2 cc.	1st	24	28	30	All injections were administered intraperitoneally; antigen and test culture
		24			
	2nd	36	36	36	
			36	S	
	3rd	42	S	S	The surviving mice were subsequently injected with virulent Type I culture in corresponding doses. All died (within the same period that control mice died)
			42	S	
	4th	60	S	60	
			S	S	
	5th	S	S	6S	
			S	S	
Filtrate of bacteria 0.4 cc.	1st	24	28	S	All antigens were prepared from an 11 hr. Pneumococcus Type II culture
			28	30	
	2nd		40	40	
			40		
	3rd	42	40	40	
			40	43	
	4th	S	S	S	
			S	S	
	5th		S	S	
		S	S	6S	
Washed bacteria 0.2 cc.	1st	24	28	30	
			28	S	
	2nd	36	40	40	
			40	40	
	3rd	42	40	40	
			40	43	
	4th	S	62	62	
			S	S	
	5th	S	S	S	

Controls	.0001	.00001	.000001	.00001	.000001	.0000001
	.01	.001	.0001	.00001	.000001	.0000001
	15	15	25	35	6S	S5

As noted in Table I no protection was evidenced in any of the vaccinated animals on the 1st and 2nd days.   Immunity began on the 3rd day in the case of the animals who received the serum vaccine and on the 4th day in those who received the washed bacteria vaccine and the filtrate of the shaken bacteria.   The immunity was furthei increased on the 5th day.   The mice who survived from this experi ment were injected with a virulent Type I culture in corresponding doses to the Type II culture.   All died.

Active immunity is therefore demonstrated on the 3rd day after injection of the serum vaccine, and on the 4th day in the case of the

TABLE II.

Active Immunity in Mice after Injection of 8 Hour Serum Vaccine.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture				Remarks
		.001	.00001	.000001	.0000001	
Pneumococcus II 8 hr. serum vaccine 400 million organisms	1st	40	40	60	60	Vaccine and test culture administered intraperitoneally
	2nd	60	40	40	24	
	3rd		S	S	90	
	4th		S	S	S	
	5th		S	S	S	
	6th	40	S	S	21	
	7th	S	40	S	S	
Controls		40	21	40	96	

washed bacteria vaccine and the filtrate of the shaken bacteria.   It is progressive in degree and specific for type.   The failure of any of the vaccinated animals to survive an injection of Type I culture indicates that the protection against Type II culture was not due to increase in general resistance but rather to type-specific immunity.   This was produced both by the use of the intact cell and by the water-soluble specific substance shaken off the bacteria.

Experiment 2.—(Table II.)   An 8 hour serum vaccine was injected daily for 7 days into a series of white mice, four mice to each day.   The dose was 0.4 cc., representing 400 million organisms, administered intraperitoneally.   The test culture was of moderate virulence, 10–6 cc. being fatal to a mouse.

As shown in Table II, immunity is present from the 3rd to the 7th days inclusive.

Experiment 3.—(Table III.) An 8 hour serum vaccine was injected daily for 5 days into a series of white mice, five mice to each day. The dose was 0.2 cc.,

TABLE III.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of Low Virulence.

Antigen used and dose	Day after vacci-nation	Survival after injection of Pneumococcus II culture					Remarks
		.01	.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 100 million organisms	2nd	S	S	S	S	S	Pneumococcus culture of relatively low virulence, 10-6 surviving and 10-5 fatal in 130 hrs.
	3rd	S	S	S	S	S	
	4th	S	S	S	S	S	
	5th	S	S	S	S	S	
Controls..................		36	22	36	130	S	

TABLE IV.

Active Immunity in Mice after Intraperitoneal Injection of 8 Hour Serum Vaccine. Test Culture of High Virulence.

Antigen used and dose	Day after vacci-nation	Survival after injection of Pneumococcus II culture				Remarks
		.001	.0001	.00001	.000001	
Pneumococcus II 8 hr. serum vaccine 200 million organisms	1st	14	44	44	44	Pneumococcus culture at maximum virulence, fatal in 10-7 cc.
	2nd	20	20	20	44	
	3rd	44	44	20	44	
	4th	20	53	S	S	
	5th	53	S	S	20	
Controls.....................		14	14	20	20	

representing 200 million organisms. The test culture was of low virulence, 10-5 cc. being fatal in 130 hours. Immunity was demonstrated from the 2nd to the 5th day inclusive.

Experiment 4.—(Table IV.) A similar experiment to the above was performed, employing however, a highly virulent test culture, 10-7 cc. being fatal to a mouse. Immunity was obtained on the 4th and 5th days only.

TABLE V.

*Passive Immunity in Rabbits after Intravenous Injection of (1) Serum Vaccine,
(2) Berkefeld Filtrate of Shaken Bacteria, (3) Washed Bacteria
Vaccine, (4) Berkefeld Filtrate of Broth Culture.*

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	.001	.0001	.000001	
Rabbit 4 pneumococcus II 11 hr. serum vaccine 5 billion organisms	3rd	S	60	S	18	45	45	Mice employed in Pneumococcus I infections were used previously in Pneumococcus II experiments in which they survived injections of virulent Pneumococcus II culture
			S	S		45	45	
	5th	S	S	S	45	45		
			S	S				
	7th	S	S	S				
			S	S				
	9th	S	42	S				
			42	S				
	15th	S	S	S				All rabbit injections given intravenously
			S	S				
	20th	42	S	S				
			S	S				
Rabbit 7 pneumococcus II filtrate of shaken bacteria 8 cc.	3rd	42	42	42				Concentration of antigens— 1 billion organisms to 1 cc. distilled water
			42	60				
	4th	42	42	42				
			60	48				
	5th	S	S	S	42	45	45	
			S	S		45	45	
	6th	S	S	S	45	45	45	
			S	S		45	45	
	8th	S	S	S				
			S	S				
	11th	S	S	S				
			S	42				
Rabbit 5 pneumococcus II washed bacteria vaccine 5 billion organisms	3rd	S	42	S	45	45	45	
			S	S		45	45	
	5th	S	S	S	45	45	45	
			S	S		45	45	
	7th	S	S	S				
			S	130				
	9th	S	S	S				
			S	S				

TABLE V—*Concluded*.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus II culture			Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	.001	.0001	.000001	
Rabbit 6 Pneumococcus II filtrate of broth culture 10 cc.	3rd	36	S	60				
			S	60				
	4th	36	42	36				
			60	S				
	5th	S	S	S				
			S	S				
	7th	S	S	S				
			S	S				
	15th	S	S	S				
			S	S				
	20th	S	S	S				
			110	S				
Rabbit serum	Before	42	42	42				
Controls Pneumococcus II			42	42				
Controls Pneumococcus II		24	24	24				
Controls Pneumococcus I		48	48	60				

The foregoing experiments indicate that the onset of active immunity alters with the virulence of the test culture, appearing on the 2nd day after vaccination when an organism of low virulence is used, on the 4th day with an organism of very high virulence, and on the 3rd day with an organism of moderately high virulence. Varying the virulence of the organism represents the progressive increase in active immunity from the 2nd to the 5th day better than varying the number of organisms injected, for a highly virulent culture may cause death of all the animals even in high dilutions and a culture of low virulence may result in complete survival. This is in accordance with the results of Neill and Gaspari (28) who found that within a certain range (10^{-6} to 10^{-3} cc. of culture) the number of invading bacteria was without influence in determining the occurrence of infection and fatality.

Experiment 5.—(Table V.) Four antigens were used in this experiment: (1) 11 hour serum vaccine, (2) filtrate of shaken bacteria, (3) washed bacteria vaccine, (4) filtrate of broth culture. The dose of the serum vaccine and the washed bacteria vaccine was 5 billion organisms, the filtrate of the broth and the shaken bacteria was in each case derived from 10 billion organisms. Each antigen was administered intravenously to a rabbit, and blood taken, before and from the 3rd to the 9th day or longer. The mouse protection test was carried out according to the technique of Dochez (25). Thus, 0.2 cc. of rabbit serum was mixed with

TABLE VI.

Passive Immunity in Rabbits after Injection of (1) Serum Vaccine and (2) Berkefeld Filtrate of Shaken Bacteria.

Antigen used and dose	Day after vacci- nation	Survival after injection of Pneumococcus II culture				Remarks
		.01	.001	.0001	.00001	
Rabbit 3 Pneumococcus II serum vaccine 2 billion organisms	1st	17	64	64	64	All antigens admin- istered intravenous- ly. Vaccine and fil- trate made from 8 hr. serum broth culture
	2nd	30	30	30	64	
	3rd	30	S	S	S	
	4th	S	S	S	S	
	5th	50	30	S	S	
	6th	17	S	S	S	
	7th	S	S	S	S	
Rabbit 1 Pneumococcus II serum vaccine 2 billion organisms	3rd	64	64	S	S	
	4th	30	S	S	S	
	5th	46	S	S	S	
Rabbit 2 Pneumococcus II filtrate of shaken bac- teria 2 cc.	4th	72	S	S	S	
	5th	S	S	S	S	
Controls....................		30	22	36	130	

varying dilutions of culture and injected intraperitoneally into mice. Survival in these as in the previous active immunity experiments is recorded when the mouse lives 6 days after injection. The blood drawn on the 3rd, 5th, and 6th days was also mixed with varying dilutions of Type I culture to determine whether the early immunity secured was a general non-specific increase in natural resistance or was entirely due to the development of Pneumococcus Type II specific protective substance. The test culture was of moderately high virulence, 10–6 cc. killing control animals as well as animals who received the serum of normal rabbits (*i.e.*, before their vaccination with pneumococcus antigen.)

As Table V indicates, the onset of passive immunity was on the 3rd day in the case of the serum vaccine and the washed bacteria vaccine. The broth filtrate and the filtrate of the shaken bacteria show a definite immunity on the 5th day. The immunity persists as long as observations in the experiments were conducted, from the 11th to the 20th day after injection. In a similar experiment noted in Table VI, the serum vaccine initiates passive protection on the 3rd day also, but the filtrate of the shaken bacteria produces an immune response on the 4th day. The rabbit sera of the animals whose blood protected against Type II culture failed to show any protection

TABLE VII.

Passive Immunity in Rabbit after Intravenous Injection of Serum Vaccine.

Antigen used and dose	Day after vacci- nation	Survival after injection of Pneumococcus II culture				Remarks
		.001	.00001	.000001	.0000001	
Rabbit 1 Pneumococcus II 8 hr. serum vac- cine 2 billion organ- isms	2nd	19	19	48	48	Virulence of pneumococ- cus culture was at its maximum, fatal in 10-7 cc.
	3rd	19	22	48	19	
	4th	19	48	48	49	
	5th	48	48	48	22	Mouse with immune horse serum was not protected against 10-6 culture.
	6th	19	48	72	72	
Immune serum		S	S	19	S	
Controls...................		22	48	48	72	

against Type I culture, demonstrating that the passive immunity produced was specific for type and not dependent upon increase of general resistance or on the common protein antibody.

Experiment 7.—(Table VII.) An 8 hour serum vaccine was injected intra-venously to a rabbit, and blood drawn from the 2nd to the 6th day inclusive. The dose was 2 billion organisms. The test culture was at maximum virulence, 10-7 cc. being regularly fatal.

No animals survived in this experiment. The blood of rabbits varies to an extent in its power to elaborate protective substance, particularly in the case of a culture of maximum virulence.

Experiment 8.—(Table VIII.)   A series of mice were inoculated intraperitoneally with an 11 hour (1) serum vaccine Type I pneumococcus and (2) filtrate of the shaken bacteria.   The dose of the vaccine was 0.2 cc., representing 200 million organisms, and of the filtrate 0.4 cc., representing 400 million organisms.   The test culture was of very high virulence, 10–7 cc. being fatal to a mouse.

Active immunity began on the 4th day in the animals vaccinated with the serum vaccine, and on the 5th day with the animals vaccinated with the filtrate of the shaken bacteria.   This indicates again that the use of a test culture of very high virulence records the onset

TABLE VIII.

Active Immunity Following Intraperitoneal Injection of Type I Vaccine.

Antigen used and dose	Day after vacci- nation	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.000001	
Pneumococcus I 11 hr. serum vaccine 400 million organisms	1st	20	20	24	Vaccine and test culture administered intraperitoneally
			20	36	
	2nd	20	20	24	
		36	36	36	
	3rd	24	36	36	
			36	36	
	4th	36	S	S	
			S	48	
	5th	S	S	S	
			S	48	
Controls......................		20	24	36	
			20	24	

of immunity at a later date than one of lesser virulence, and thus registers the progressive increase in antibody response from the 3rd to the 5th day.

Experiment 9.—(Table IX.) In this experiment two antigens from Pneumococcus Type I were employed, (1) the Berkefeld filtrate of the shaken bacteria and (2) the filtrate of the broth culture. A comparison was made between the standard dose and a very large dose.   A series of mice were inoculated intraperitoneally with 0.4 cc. of broth filtrate, a second series with 1.0 cc. of broth filtrate, a third series with 0.2 cc. of the filtrate from the shaken bacteria, and a fourth with 1.0 cc. of the filtrate of the shaken bacteria.   Each antigen was injected daily into eight mice, and on the 7th day after the first injection, the test culture was administered to the

entire series, comprising mice injected 2 to 7 days after inoculation with antigen. The test culture was of high virulence, 10-7 being fatal to a mouse in 60 hours.

As will be seen in Table IX, all the mice injected on the 2nd day after vaccination died, 32 in number. Survival began on the 3rd day after injection in each series but usually in only one out of eight animals. In the case of the filtrate of the shaken bacteria, definite protection was present on the 4th day, increasing to its height on the

TABLE IX.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intra-peritoneal Injection of (1) Berkefeld Filtrate of Shaken Bacteria, (2) Berkefeld Filtrate of Broth Culture.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Broth filtrate 0.4 cc.	2nd	40	40	40	58			All injections administered intraperitoneally. Filtrates made from 11 hr. serum vaccine, Pneumococcus Type I.
		40	58	58	58			
	3rd	58	58	58	40			
		58	58	58	S			
	4th	18	40	58	58			
		58	58	58	S			
	5th	40	40	58	40			
		58	58	S	S			
	6th	40	40	58				
		40	80	S	S			
	7th	40	S	58	S			
		40	S	S	S			
Broth filtrate 1.0 cc.	2nd	40	40	40				
		40	58	58	58			
	3rd	40	40	40	40			
		40	40	S	58			
	4th	40	40	58	S			
		58	S	58	S			
	5th	40	40	40	S			
		58	40	58	80			
	6th	40	48	58	S			
		40	58					
	7th	40	58	S	S			
		40	S	S	S			

TABLE IX—*Concluded*.

Antigen used and dose	Day after vaccination	Survival after injection of Pneumococcus I culture						Remarks
		.01	.001	.0001	.00001	.000001	.0000001	
Bacterial filtrate 0.2 cc.	2nd	18	40	40	58			
		40	40	40	58			
	3rd	40	40	40	40			
		40	S	40				
	4th	40	40	40	S			
		40	40	S	S			
	5th	18	S	S	S			
		40	80	S	S			
	6th	40	58	80	S			
		S						
	7th	40	S	S·	18			
		40	S		18			
Bacterial filtrate 1.0 cc.	2nd	40	40	40	58			
		40	40	58	58			
	3rd	40	40		S			
		40	40	40				
	4th	40	40					
		40	80	S	S			
	5th	40	18	S	S			
		40	S	S	S			
	6th	40	40	S	S			
		58	58					
	7th	40	58	40	S			
		40	58	58				
Controls.............		40	40	58	58	58	60	
		40	40		58			

5th day, and remaining approximately stationary to the 7th. In fact, in this experiment there was a slight falling off in survival from the 5th to the 7th day. The increased dose (1.0 cc. of filtrate) gave slightly inferior results than the standard dose (0.2 cc.).

The immunity resulting from the standard broth dose (0.4 cc.) was less marked than that from the filtrate of the shaken bacteria. Animals survived from the 3rd to the 6th day in small numbers and on the 7th day, five out of eight survived. The large dose of broth (1.0 cc.) gave a less marked immunity response but here too, five out of

TABLE X.

Effect of Variation in Dosage of Antigen on Active Immunity in Mice after Intra-peritoneal Injection of Serum Vaccine.

Antigen used and dose	Day after vacci-nation	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
Serum vaccine dose 0.2 cc.	2nd	44	44	70	All injections administered intraperi-toneally
		44	44	70	Vaccine grown for 8 hrs. in 2.5 per cent
		44	44	44	serum broth
	3rd	92	92	SS	Numbers in table refer to length of
		SS	SS	S	time in hrs. which mice lived after
	4th	SS	SS	SS	injection. S indicates survival for
		S	S	S	6 days or more
	5th	SS	SS	SS	
		S	S	S	
Dose 0.02 cc.	2nd	44	44	44	
		44	44	44	
			44		
	3rd	44	44	44	
		44	44		
		S	S	SS	
	4th	SS	92	SS	
		S	SS	S	
	5th	92	SS	SS	
		SS	S	S	
Dose 0.002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	SS	44	SS	
			92		
		S	S	S	
	4th	SS	SS	SS	
		S	S	S	
	5th	SS	SS	SS	
		S	44	S	
Dose 0.0002 cc.	2nd	44	44	44	
		44	44	44	
		44	44	44	
	3rd	44	SS	44	
		44		44	
		S	S	S	
	4th	SS	44	92	
		S	SS	SS	
	5th	44	70	92	
		92			
		S	SS	SS	

Normal controls........		.001	.0001	.00001	.000001	.0000001	.00000001	.000000001
		44	44	44	44	44	44	44

eight survived on the 7th day. It is observed that in only one instance (1 of 192 mice), did an animal resist 0.01 cc. of test culture. On the 5th day, however, the filtrate of the shaken bacteria produced an immunity sufficient to withstand an injection of 0.001 cc. of a culture with a virulence of 10^{-7}.

Experiment 10.—A series of mice were injected intraperitoneally with the following doses of Type I vaccine; (1) 0.2 cc., (2) 0.02 cc., (3) 0.002 cc., (4) 0.0002 cc.

TABLE XI.

Active Immunity after Intraperitoneal Injection of Pneumococcus Type I Culture to Which Sodium Ricinoleate Had Been Added.

Antigen used and dose	Day after vacci-nation	Survival after injection of Pneumococcus I culture			Remarks
		.001	.0001	.00001	
0.1 per cent sodium ricinoleate Pneumococcus Type I culture	1st	44	20	44	11 hr. Pneumococcus Type I broth culture exposed to a final concentration of 0.1 per cent sodium ricinoleate before injection
		44	44	44	
	2nd	44	44	44	
		44	44	44	
	3rd	44	20	44	
		44	44	84	
	4th	44	44	S	
		S	44	S	
Dose 0.4 cc.	6th	S	S	S	
		S	S	S	
	7th		44	44	
		S	S	S	
Dose 0.04 cc.	1st	20	20	20	
		20	20	20	
	4th	44	44	S	
		64	64	S	
Normal controls............		.001 44	.0001 44	.00001 44	.000001 44	.0000001 44	.00000001 44

1 cc. of the vaccine contained 5 billion organisms. Twelve mice were inoculated daily with each antigen, and on the 5th day a test culture of Pneumococcus Type I was injected intraperitoneally into the entire group. The virulence of the culture was such that 10^{-9} cc. was fatal to a control animal.

As seen in Table X all the 2nd day mice (48 in number) as well as all the control animals died. Survival began on the 3rd day and was

more complete on the 4th and 5th days.   The immunity produced was fairly constant in the range of dosage between 0.2 cc. and 0.002 cc., but began to diminish when the dose of vaccine was lowered to 0.0002 cc.   The degree of protection on the 3rd day was sufficient to protect against 100,000 minimal lethal doses.   (When the maximal virulence of the organism 10^{-9} is used in the calculations, the protection is equivalent to 1 million minimal lethal doses.)   The Type I organism produces a more marked early immunity than the Type II.

Experiment 11.—(Table XI.)   The antigen used in this experiment was a virulent Type I culture exposed to a final concentration of 0.1 per cent sodium ricinoleate. Mice were inoculated with this culture intraperitoneally daily for 5 days, dose 0.2 cc. On the 5th day a virulent test culture was injected intraperitoneally.

Survival began on the 4th day, and was complete on the 6th.   The use of sodium ricinoleate did not hasten the immunologic response. The total results were not as good as those which were produced by the use of the serum vaccine.

DISCUSSION.

The experiments reported in the present paper are concerned mainly with (1) the character of the pneumococcus antigen as a factor in the antibody response, (2) the onset and rate of development of immunity to the pneumococcus.

As reviewed in the early part of this paper, some observers have found the intact bacterial cell necessary for the development of type-specific immunity, whereas others have reported that solutions or extracts of the pneumococcus are adequate for this purpose.   We have employed four antigens, two of them consisting of the intact cell, namely, the serum vaccine and the washed bacteria vaccine; and two of them containing water-soluble substances derived from the cell, namely, a Berkefeld filtrate of the shaken bacteria and a filtrate of the broth culture.   The latter antigens are contained in a clear water solution free from formed elements.

Mice were actively immunized by a single intraperitoneal injection of each antigen.   On the 5th day after the injection a high grade type-specific immunity was demonstrated in all.   Survival of the mice to a virulent culture of the homologous organism was not deemed sufficient

to prove type-specific immunity, for it appeared possible that the increased resistance might be due to augmenting the natural defensive mechanisms or to the stimulation of the common protein antibody. Tillett (26) in this connection found that immunization of rabbits with Type I or Type II pneumococci, and with R forms derived from any of the fixed types, was equally effective in producing active immunity against Type III infection. All the mice, therefore, that survived injection of Type II culture after previous vaccination with Type II antigen were injected with virulent Type I culture. None survived. Wright (27) was also unable to demonstrate increased resistance to Type I pneumococci by previous injection with heterologous organisms.

After a single intravenous injection to rabbits of each of the four antigens described above, all sera on the 5th day showed type-specific protective substance. The sera which protected mice against Type II culture gave no protection against Type I culture. Thus, in both active and passive immunity experiments a Berkfeld filtrate containing a watery extract of the pneumococcus cell resulted in a type-specific antibody response.

The onset of definite type-specific immunity, both active and passive, appeared on the 3rd day after injection of the serum vaccine. It increased progressively to the 5th day and remained approximately unchanged to the 7th day or longer. The washed bacteria vaccine resulted in a similar slightly less marked response. The immunity resulting from the filtrate of the shaken bacteria and the broth filtrate began on the 4th day after injection, increased on the 5th, and remained stationery from the 5th to the 7th day. The degree of active immunity secured on the 5th day from Pneumococcus Type II vaccine was approximately that which protected a mouse from an intraperitoneal injection of 0.001 cc. of a culture with a virulence of 10-6 cc. In the case of Type I vaccine protection was obtained against 0.001 cc. culture with a virulence of 10-7 cc. The degree of antibody response cannot be measured accurately solely by recording survival after injection of varying doses of test culture, for as pointed out earlier in the paper and as observed by Neill and Gaspari, the incidence of infection between the range 10-3 and 10-6 is not wholly dependent upon dilution. We have considered as of almost equal

importance the total number of animals surviving of the entire group (10-3 to 10-6) irrespective of the dose administered.   Lastly, varying the virulence of the organism was of especial help in determining the graded increase of early immunity.   When active immunity was tested against a Pneumococcus Type II organism of moderately high virulence, (10-6), well marked specific immunity appeared on the 3rd day.   With an organism of still higher virulence (10-7) an immune response was noted on the 4th day.   In two instances, when a test culture of low virulence was employed, evidence of active immunity appeared on the 2nd day.   (Test culture was fatal to a mouse, 10-5 cc. in 130 hours.)   In the case of Type I pneumococcus vaccine, active immunity appears on the 3rd day even against a highly virulent 10-7 organism.

By evaluating these three criteria of resistance, we feel justified in stating that an antigen derived from the intact cell gives rise to a type-specific immunity both active and passive beginning definitely on the 3rd day, increasing to the 5th, and remaining approximately stationery to the 7th or longer.   The antigen contained in the filtrate, free from formed elements, initiates a type-specific immunity on the 4th day, increases markedly to the 5th, and remains approximately stationery to the 7th day.   The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II.   On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses.   On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

We are unable at this time to evaluate the factors which are responsible for the early induction of immunity to the pneumococcus.   In the preparation of our vaccine we have employed highly virulent cultures, relatively short incubation periods, and the addition of human serum to the broth.   The final vaccine was cleared of broth by carefully rinsing the centrifuge tube containing the sedimented bacteria instead of washing, since the washings contained considerable highly antigenic material.   The average dose of vaccine administered to the rabbit was 2 to 5 billion organisms intravenously; to the mouse 200 to 400 million organisms intraperitoneally.

This study was undertaken with the object of determining whether

an active immunity to the pneumococcus could be established in a sufficiently short space of time as to make the injection of vaccine a therapeutic possibility in lobar pneumonia. As far as the time interval is concerned, our results support this hypothesis. Whether the patient with lobar pneumonia would react as experimental normal animals do raises a question not within the scope of this paper.

<div align="center">CONCLUSIONS.</div>

1. The antigenic function of a pneumococcus vaccine made from the intact cell was compared with that derived fron a watery extract of the cell free from formed elements. In each instance, the immunity produced was dependent upon type-specific protective substance and not upon the elaboration of the common protein antibody.

2. The vaccine made from the intact cell resulted in both active and passive immunity which began on the 3rd day, increased markedly to the 5th, and remained approximately stationery to the 7th day. In the case of the Berkefeld filtrate of the shaken bacteria and the filtrate of the broth culture, the immunity began on the 4th day, increased to the 5th, and remained approximately stationery to the 7th day. The immunity produced by Pneumococcus Type I vaccine is greater than that produced by Type II. On the 3rd day, mice vaccinated with Type I vaccine resisted 100,000 minimal lethal doses, whereas mice immunized with Type II resisted 10,000 minimal lethal doses. On the 5th day, a larger percentage of mice survived these doses than on the 3rd day.

3. Certain factors related to the preparation and dosage of the vaccine are discussed.

4. As far as the time interval and the degree of immunity produced are concerned, these results suggest the possibility of employing pneumococcus vaccine in suitable doses in the treatment of lobar pneumonia. That an earlier activity of the immunity mechanism could actually be initiated in a patient with lobar pneumonia has still to be demonstrated.

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THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

III. The Malignant Fibroblast of Sarcoma 10 of the Crocker Foundation.

By ALEXIS CARREL, M.D., AND ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 4.

The definition of a cell by its morphological characteristics, according to classical cytology, is of an unsatisfactory nature because the individuality of an anatomical element depends more on its physiological properties than on its appearance.[1] A description of a given structure remains almost without significance if the relations that correlate its form and function remain unknown. Such a study should always be completed by a thorough investigation of the fundamental physiological properties of the element considered. In this manner, the individuality of the fibroblast and the macrophage has become clearly defined;[2,3] but an investigation of the sort must be undertaken for every cell type that has been obtained in pure culture. If a similar study were made of the cells composing experimental tumors, the nature of malignancy might soon be discovered. A search has already been started for the essential characteristics of the specific elements of Rous sarcoma,[4] of rat sarcomas,[5] and of mouse and rat carcinomas,[6] with the object of ascertaining the factors which cause

[1] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1198. Carrel, A., in Cowdry, E. V., Special cytology, New York, 1928, 1.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 261.

[3] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 285.

[4] Carrel, A., *Compt. rend. Soc. biol.*, 1925, xcii, 584.

[5] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1119.

[6] Fischer, A., *Z. Krebsforsch.*, 1927, xxv, 89. Laser, H., *Z. Krebsforsch.*, 1927, xxv, 298.

these cells to multiply indefinitely within the organism.   The purpose of the present paper is to describe the properties that distinguish the malignant fibroblast of Sarcoma 10 of the Crocker Foundation from the normal fibroblast of the rat, when both cell types are living in pure cultures.

Isolation of a Strain of Malignant Fibroblasts.

Rat Sarcoma 10, which was obtained by us from the Crocker Foundation through the kindness of Dr. F. C. Wood, is a tumor easily transmissible by transplantation.   Its growth is rapid.   It spreads locally, reaches a very large size, and kills the animal by cachexia.   There are no metastases.   Regression of the tumor is exceptional.   It is composed of large, short spindle cells with oval nuclei and one or two nucleoli, densely packed together.   Between the cells are scattered ˜ many macrophages which may easily be distinguished from the fibroblasts by the appearance of their nucleus.   When a pure culture of malignant fibroblasts is inoculated into a rat, the tumor which develops after less than 5 days already shows many macrophages mixed with the spindle cells.   The macrophages thus appear to be a normal constituent of the tumor.

In November, 1926, a few small fragments of Sarcoma 10 were cultivated in D flasks containing a solid medium composed of chicken plasma.   When chicken plasma is diluted with 3 volumes of Tyrode solution, coagulated with 1 drop of chick embryo juice, and washed once or several times in an excess of Tyrode solution, it yields a coagulum which is not toxic for foreign cells.[7]   This coagulum has the advantage of remaining transparent and of not being digested by the rat tissues.

Some diluted chick embryo juice was injected at the surface of the coagulum as a nutrient medium, for it is known that rabbit, guinea pig, rat, fowl, and other animal tissues, utilize foreign embryo proteins for the building up of new protoplasm.[8]   During the first 24 hours of incubation, the tissue fragments surrounded themselves with a crown of macrophages.   The migration of these macrophages was more abundant in a nutrient medium composed of heparinized rat plasma or of rat serum, than when embryonic juice was used.   In rat serum or heparinized plasma, the macrophages spread rapidly

[7] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 601.

[8] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 603.   Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 499.

throughout the medium. But in a nutrient medium composed exclusively of chick embryo juice, they grew very much less extensively, while the fibroblasts rapidly increased in number. Every 2 or 3 days, after the cultures had been washed in Tyrode solution, the nutrient medium was changed. When the tissues had grown for about 10 days, the solid medium was removed from the flask, spread on a glass plate, and the area of growth isolated by four sharp cuts of a cataract knife. It was then divided into two parts and placed in a fresh flask. After a few passages in embryo tissue juice, all the macrophages disappeared, and a pure strain of large, short fibroblasts, similar to those seen in the sections of the tumor, was obtained. The morphology of the cells varied considerably according to the medium. They often appeared as long and densely packed fibroblasts which invaded the solid medium as a thick tissue. The rate of growth was measured by the ordinary technique.[9]

From time to time, a few fragments of cultures were inserted in the subcutaneous connective tissue of rats. 4 or 5 days after inoculations made in December, 1926, when the strain of fibroblasts was already free from macrophages, a small tumor appeared at the site of injection, grew rapidly, and eventually killed the animals. The fibroblasts evidently carried the malignant characteristics. Similar experiments were done later, with identical results. In January, and February, 1928, inoculations of the cultures still produced tumors within 4 or 5 days, which grew rapidly afterwards. There is, then, no doubt that malignancy is a permanent property of the strain.

A pure strain of normal fibroblasts was obtained from the rat by the ordinary technique.[9] It was cultivated under the same conditions as the sarcomatous cells, and used as a means of comparison. The morphological and physiological properties of the normal and malignant strains were studied in the same manner as those of the normal chicken fibroblasts described in a previous article.[2]

Morphological Characteristics of the Cells.

The normal and sarcomatous fibroblasts were compared after they had been cultivated for a few days in identical media, and when their

[9] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

rates of growth had become similar. Such preliminary conditions were necessary since it has been found that the nuclear and proto-·plasmic structures of normal fibroblasts vary in a large measure according to their metabolic state.[2] Were the functional state of the cells not ascertained, phenomena due to nutritional or degenerative changes might be falsely attributed to pathological factors.

In the following experiments, the normal and sarcomatous cells respectively were cultivated for several days in a medium composed of embryo tissue juice. Then, a few fragments of the cultures were transferred to hanging drops of plasma and embryonic juice. From 1 to 48 hours after the cover glasses were prepared, the tissues were stained with 1/20,000 Janus green and 1/50,000 neutral red. Camera lucida drawings were made of the cells at a magnification of 1,600 to 3,200 diameters.

The normal fibroblasts of the rat closely resembled the chicken fibroblasts previously described.[2] They were elongated cells with sharp boundaries and processes open at the end. The nuclei were long and oval, with one or two nucleoli. In about 0.5 per cent of the cells, two or three nuclei were observed. The dimensions of the cells averaged approximately $30 \times 115\mu$, and those of the nuclei $8.5 \times 19\mu$. The projected areas of the cell and nucleus were, respectively, 1,960 and 189 sq. μ. The segregation apparatus was small, and localized in the forward part of the cytoplasm around the centriole. Long filamentous mitochondria were seen around the nucleus and within the processes (Fig. 1, A).

The malignant cells were generally larger and coarser than the normal ones, and the cytoplasm was more refringent. Their length was about 125μ, and their width 39μ. The nucleus was globular, $12 \times 18.8\mu$, and wider but a little shorter than that of the normal cells. The projected areas of the cell and nucleus were, respectively, 2,300 and 230 sq. μ. The segregation apparatus was very small. There were no degenerative vacuoles or abnormal mitoses. The mito-chondria were similar to those of normal cells (Fig. 1, B). Multinu-clear cells were present in the proportion of about 0.5 per cent. Some of these cells contained a large number of small nuclei. However, the percentage of the abnormalities did not exceed that present in normal cultures. The malignant fibroblasts apparently did not de-

generate or die when cultivated as a pure strain. They looked like healthy cells distinguished from normal fibroblasts merely by their size and the particular appearance of their cytoplasm. Sarcomatous and normal fibroblasts may indeed assume, in some cultures, an identical form. So far, no morphological characteristic has been discovered which can be considered as specific of malignancy.

The cinematographic records of normal fibroblasts of the rat showed that their mode of locomotion is identical with that of chicken fibroblasts. The cells generally move out from the center of the colony in a straight line. Their activity is polarized, the outer pole being characterized by the presence of the centriole. The protoplasm streams through the apparently rigid walls of the open end of the front process. Then the nucleus and the cytoplasmic organs glide forward, dragging the rear process. Sarcomatous fibroblasts showed no essential differences, even when both normal and malignant strains were cultivated in the same medium and cinematographed simultaneously.

Architecture of the Colonies.

Normal fibroblasts never grow as isolated units. They do not scatter through the medium as macrophages do. When they migrate into the surrounding coagulum, they remain in intimate reciprocal contact on all sides and multiply actively when packed together. A fibroblast colony always forms a dense tissue. This characteristic establishes a fundamental difference between fibroblasts and macrophages. Macrophages live as independent units and die if they congregate in masses. Normal rat fibroblasts form round or oblong colonies, which are similar in appearance to those of chicken fibroblasts. They never invade the entire medium. When a small colony of rat fibroblasts is cultivated in a washed coagulum of chicken plasma in a D flask, and fed upon diluted chick embryo juice at a pH of about 7.4, it reaches a diameter of about 8 or 10 mm. in 5 or 6 days. Later, the rate of growth decreases and the cells have a tendency to degenerate. Under the present conditions, in order to keep its activity, the strain must be transferred to a fresh coagulum at the end of 6 days. The limitation in the size of the colonies is probably due to the same cause which also prevents chicken fibroblasts from invading the entire medium. In the colonies of rat fibroblasts, a thick center forms more often than is the case with

chicken fibroblasts, and degeneration begins unless the necrotic tissue is removed with a cataract knife. There is no real difference between the colonies of normal rat and chicken fibroblasts.

The colonies of sarcoma fibroblasts are similar to those of the normal type. The cells never scatter into the medium. They organize as a tissue and form round or oblong colonies. These colonies are thicker than those of normal fibroblasts and are easily recognized on account of this trait. They also reach a greater size, as they actively invade the medium for 10 or 12 days. Then, their diameter may be 15 or even 20 mm. They differ from the colonies of normal fibroblasts by their large surface and their greater opacity, and not by their architecture.

Cultures of normal and of sarcomatous fibroblasts which were placed side by side in a flask exchanged cells freely. Cinematograph records taken at the beginning of the symbiosis clearly showed that normal cells were not repelled or destroyed by the malignant ones. At this time, there was no morphological difference between the two types of cells. After a few days, composite colonies were obtained made up of normal and tumor tissues. These colonies were divided and transferred to flasks, and both types of cells, which had become easily distinguishable morphologically, were observed to migrate into the medium. But after some time, the sarcomatous tissue progressively invaded the normal tissue, which ultimately disappeared almost completely.

Residual Growth Energy.

It is well known that the inherent growth energy of a fragment of fresh tissue or of a pure culture of tissue cells can be measured by its residual energy,[10] that is, by the duration of the life of the cells and the activity they display when deprived of nitrogenous food in a medium composed of Tyrode solution. The residual energy of tissue cells probably depends on their capacity for accumulating food material while being cultivated in a nutrient medium. A comparison was made between the residual energy of colonies of normal and sarcomatous fibroblasts after they had lived for some weeks in embryonic tissue juice. Six experiments were performed, as reported in Table I.

[10] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 521.

Text-fig. 1 expresses the results of an experiment which is typical. The duration of life of both normal and sarcomatous fibroblasts in Tyrode solution did not exceed 6 days. The relative increase of the normal colonies was slightly greater than that of the sarcomatous ones. The residual growth energies of both cell types were approximately equal.

TABLE I.

Effect of Tyrode Solution on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Normal fibroblasts		Sarcomatous fibroblasts	
		Duration of life	Relative increase in Tyrode solution	Duration of life	Relative increase in Tyrode solution
		days		*days*	
1	1513-H	5	0.95	5	1.40
2	1513-H	5	2.39	5	1.80
3	1517-H	9	4.30	9	1.58
4	6499-C	5	2.96	5	2.15
5	6499-C	5	3.45	5	3.07
6	6512-C	4	2.44	5	1.23
Average...............		5.5	2.75	5.67	1.87
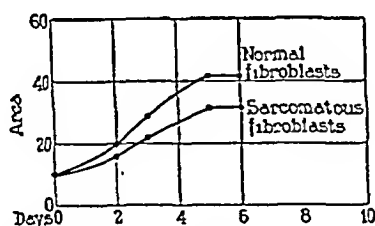
TEXT-FIG. 1. Experiment 6499-C. Residual activity of normal and sarcomatous fibroblasts of the rat.

Duration and Rate of Growth.

When cultivated in a nutrient medium, normal rat fibroblasts multiplied in an unlimited manner, as chicken fibroblasts do. After about 16 months of life *in vitro*, sarcomatous fibroblasts proliferated as actively as at the beginning of their period of cultivation. They had also kept their malignancy. The duration of life *in vitro* of sarcomatous and normal fibroblasts appears to be unlimited.

The rate of growth of both strains has been ascertained by the measurement of the area of the colonies, and also by the volume of the new tissue which develops in a medium composed of chick embryo juice containing about 10 mg. of nitrogen per 100 cc.   In Table II, the results of nine experiments are summarized, in which the rates of growth of normal and sarcomatous fibroblasts were compared for periods of 5 or 6 days.   The ratio of the relative increases of the colonies in nutrient and non-nutrient media was slightly larger in the case of the normal fibroblasts.   There was no fundamental difference in the rate of growth of both types of cells, as long as they were cultivated in a solution containing embryo proteins.

Effects of Normal and Sarcomatous Fibroblasts on Their Medium.

1. Liquefaction of Fibrin.—When normal and sarcomatous cells were cultivated in washed chicken plasma, no digestion of the coagulum ever occurred.   The medium always remained homogeneous. When cultivated in a coagulum of rat plasma, normal rat fibroblasts did not liquefy the fibrin (Fig. 2).   But if sarcomatous fibroblasts were cultivated in rat plasma, they always destroyed the coagulum down to the glass after 4 or 5 days (Fig. 3).   This phenomenon gave the medium an appearance similar to that of the cultures of Rous sarcoma.[11]

2. Acid Production.—It has been shown by Rous[12] that a fragment of tissue embedded in plasma rapidly modifies the adjacent medium which becomes acid to litmus.   In order to ascertain whether sarcomatous fibroblasts produce more acid than normal fibroblasts, both cell types were cultivated in a flask containing a plasma coagulum and phenol red.   This dye is less toxic than some other indicators, according to the findings of Rous,[13] and is well adapted to show the changes in the pH which might be expected to occur in such experiments. After the colonies had been embedded in 1 cc. of diluted chicken plasma, coagulated by 1 drop of embryo chick juice, and washed as usual, 1 cc. of Tyrode solution containing 0.04 per cent phenol red was injected into the flask.   After half an hour, it was removed and replaced

[11] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.
[12] Rous, P., *J. Exp. Med.*, 1913, xviii, 183; *Proc. Soc. Exp. Biol. and Med.*, 1911–13, ix–x, 161.
[13] Rous, P., *J. Exp. Med.*, 1925, xli, 451.

with embryo tissue juice containing 0.02 per cent phenol red.   After a few hours, the sarcomatous colonies appeared golden yellow, while the normal tissues were pinkish orange.   The cultures were allowed to grow for a few days.   Normal and sarcomatous fibroblasts multiplied at about the same rate.   Although they were in practically identical metabolic conditions, the sarcomatous tissues became bright yellow, while the normal colonies remained pinkish orange.   When normal and sarcomatous tissues, instead of being cultivated as separate units, were caused to live in symbiosis, a heterologous tissue made up of patches of both cells was obtained.   After the colony had been stained with phenol red, golden yellow spots appeared on a pink background. The examination of the flasks under low power showed that the yellow islands were composed of sarcomatous fibroblasts which could easily be distinguished from the normal cells by their coarser appearance. As might be expected in the light of Warburg's experiments,[14] sarcomatous fibroblasts produced much more acid than normal fibroblasts, when both cell types were placed in identical metabolic conditions. They surrounded themselves with a yellow area which extended more or less into the pink coagulum.   They appeared to thrive in a pericellular fluid more acid than that about normal fibroblasts.

Optimum H Ion Concentration of the Medium.

The optimum H ion concentration of the medium was found to differ slightly for the two cell types.   The sarcomatous fibroblasts must be cultivated in a well buffered medium at a pH of 7.5.   The normal rat fibroblasts grow better at a pH of about 7.3 to 7.4.   This slight difference is probably connected with the large quantity of acid produced by the sarcomatous fibroblasts.

Food Requirements.

When both cell types were cultivated in chick embryo juice, they multiplied indefinitely and their rate of growth was about equal, as shown in the nine experiments summarized in Table II.   The growth of the normal and sarcomatous fibroblasts of a typical experiment is expressed in Text-fig. 2.   In calf liver digest, they behaved in a differ-

[14] Warburg, O., *Naturwissenschaften*, 1927, xv, 1.

TABLE II.

Effect of Tyrode Solution and Chick Embryo Juice on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$	Period of growth	Control in Tyrode solution	Experiment in chick embryo juice	Ratio: $\frac{E}{C}$
		days				*days*			
1	6363-C					4	1.72	3.84	2.24
2	6363-C					4	1.50	2.50	1.66
3	6363-C					4	1.02	2.25	2.25
4	6425-C					5	1.25	4.03	4.03
5	6414-C	6	1.51	5.88	3.89				
6	6414-C	6	2.11	6.26	2.97				
7	6438-C	5	2.10	5.01	2.38				
8	6463-C					5	0.93	2.62	2.81
9	6463-C					5	0.90	3.42	3.80
Average.........		5.67	1.91	5.72	3.08	4.5	1.22	3.11	2.80
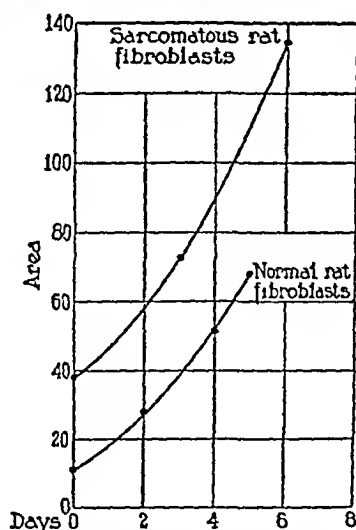
TEXT-FIG. 2. Experiments 6487-C and 9907-D. Growth of normal and sarcomatous fibroblasts of the rat in chick embryo juice.

TABLE III.

Effect of Chick Embryo Juice and Calf Liver Digest on Normal and Sarcomatous Fibroblasts of the Rat.

Experi-ment No.	Culture No.	Relative increase: Normal fibroblasts [15]				Relative increase: Sarcomatous fibroblasts			
		Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$	Period of growth	Control in chick embryo juice	Experiment in calf liver digest	Ratio: $\frac{E}{C}$
		days				*days*			
1	6418-C	5	3.22	3.66	1.13				
2	6418-C	5	3.94	3.69	0.93				
3	6418-C	5	3.14	3.97	1.26				
4	6445-C	12	3.99	3.04	0.69				
5	6445-C	13	4.85	3.65	0.75				
6	6445-C	13	3.67	3.26	0.89				
7	6420-C					8	5.00	6.50	1.30
8	6420-C					8	4.40	6.63	1.50
9	6451-C					16	4.90	5.20	1.06
10	6451-C					16	5.35	5.14	0.97
11	6488-C	7	9.20	11.02	1.20				
12	6488-C	7	8.72	8.30	1.05				
13	6479-C					21	2.04	2.22	1.09
14	6479-C					21	1.38	3.07	1.38
15	6507-C					28	5.00	6.00	1.20
16	6505-C					31	5.64	7.00	1.24
17	6505-C					31	6.17	8.31	1.34
18	6517-C	13	3.74	3.04	0.81				
19	6538-C	19	2.68	1.10	0.45				
20	6538-C	19	4.27	1.44	0.34				
21	6553-C	23	4.38	1.92	0.44				
22	6553-C	23	5.97	1.20	0.20				

[15] The figures express the relative increase of the tissue during the last passage, and not during the entire period of growth.
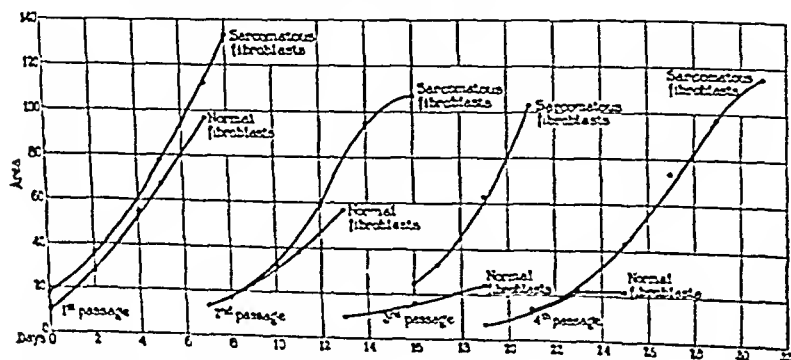
TEXT-FIG. 3. Experiments 6420-C and 6488-C. Effect of calf liver digest on normal and sarcomatous fibroblasts of the rat.

ent way.   Sarcomatous fibroblasts, studied in the twenty-two experiments summarized in Table III and in Text-fig. 3, multiplied more actively in calf liver digest than normal fibroblasts did.   The duration of their activity in calf liver digest was unlimited, while the normal cells did not multiply in the same medium for more than 3 or 4 weeks

TABLE IV.

Effect of Tyrode Solution and Chicken Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experi-ment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Dura-tion of life	Control in Tyrode solution	Experi-ment in serum	Ratio: $\frac{E}{C}$	Dura-tion of life	Control in Tyrode solution	Experi-ment in serum	Ratio: $\frac{E}{C}$
		days				*days*			
1	6477-C					5	1.32	2.00	1.51
2	6478-C					7	1.00	1.52	1.52
3	6493-C					14	1.59	1.44	0.90
4	6486-C	4	2.70	3.50	1.30				
5	6486-C					4	0.80	1.24	1.55
6	6499-C	5	3.45	3.60	1.04				
7	6499-C					5	3.07	3.04	1.10
8	6512-C	10	2.44	3.22	1.32				
9	6512-C					10	1.23	1.10	0.89
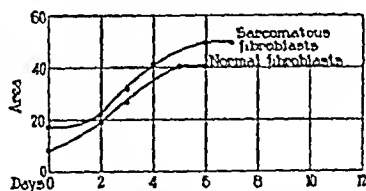
TEXT-FIG. 4.   Experiments 6486-C and 6499-C.   Effect of chicken serum on normal and sarcomatous fibroblasts of the rat.

despite a few transfers.   Calf liver digest fulfilled all the food requirements of sarcoma cells, but it failed to support the indefinite proliferation of normal rat fibroblasts.

The effect of chicken serum on both cell types was ascertained in nine experiments (Table IV).   In it the duration of life of both normal and sarcomatous fibroblasts did not exceed 8 days (Text-fig. 4).   It is

evident that the cells do not utilize chicken serum. A similar investigation was carried out in seven experiments with rat serum (Table V). Normal and sarcomatous fibroblasts did not feed on rat serum proteins. They died after less than 7 days (Text-fig. 5.) Were it not for the observations with calf liver digest, one might suppose sarcom-

TABLE V.

Effect of Tyrode Solution and Rat Serum on Normal and Sarcomatous Fibroblasts of the Rat.

Experiment No.	Culture No.	Relative increase: Normal fibroblasts				Relative increase: Sarcomatous fibroblasts			
		Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$	Duration of life	Control in Tyrode solution	Experiment in serum	Ratio: $\frac{E}{C}$
		days				days			
1	1513-H	5	0.95	1.03	1.08				
2	1513-H	5	2.39	1.90	0.80				
3	1513-H					5	1.40	2.00	1.42
4	1513-H					5	1.80	1.82	1.00
5	6506-C					5	1.60	2.10	1.31
6	1517-H	9	4.30	1.90	0.44				
7	1517-H					9	1.58	2.18	1.40
Average.........		6.33	2.55	1.61	0.77	6	1.60	2.03	1.28
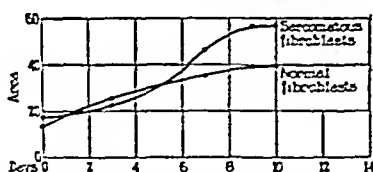
TEXT-FIG. 5. Experiment 1517-H. Effect of rat serum on normal and sarcomatous fibroblasts of the rat.

atous fibroblasts to have the same food requirements as normal fibroblasts.

Effect of Bone Marrow on Normal and Sarcomatous Fibroblasts.

In a first series of experiments, cultures of sarcomatous and normal fibroblasts were divided into two equal parts. At an equal distance from two half cultures of malignant and normal fibroblasts, a little

fragment of rat bone marrow was placed. The medium contained diluted rat serum, and no embryonic juice. The other halves of the sarcomatous and normal colonies were cultivated as controls in a flask which contained rat serum and no bone marrow. The tracings of the tissues were made under the projectoscope and the growth of each fragment was ascertained during successive days (Text-fig. 6). The results of the four experiments of this series were constant. The ameboid cells which spread from the bone marrow multiplied more abundantly around the sarcomatous than the normal colony. The growth of the sarcomatous fibroblasts became far greater than that of
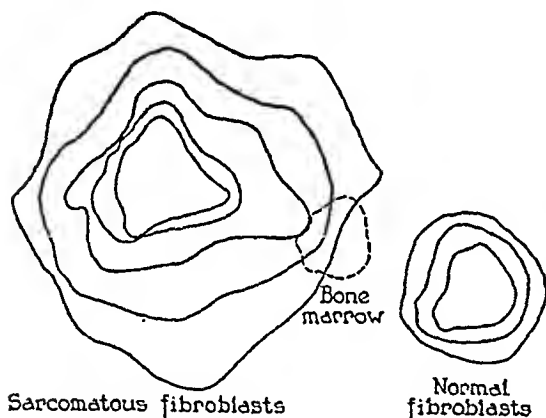
TEXT-FIG. 6.   TEXT-FIG. 7.

TEXT-FIG. 6. Experiment 1492-H. Effect of rat bone marrow on sarcomatous and normal fibroblasts of the rat in rat serum.
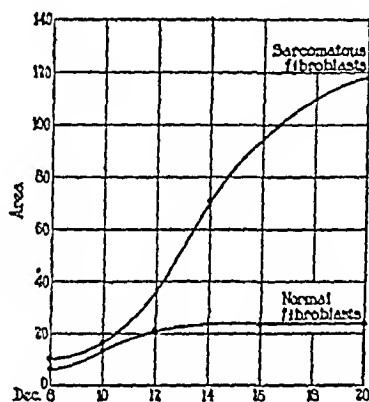
TEXT-FIG. 7. Experiment 1492-H. Effect of rat bone marrow on normal and sarcomatous fibroblasts of the rat in rat serum.

the normal (Text-figs. 6, 7). Both the controls in rat serum without bone marrow grew at equally slow rates and for only a short time. It was obvious that the multiplication of the sarcomatous fibroblasts was markedly favored by the presence of the bone marrow, while the normal fibroblasts responded but slightly. In a second series of experiments, a culture of sarcomatous fibroblasts was divided into two parts. One fragment was cultivated in rat serum without bone marrow, and the other with bone marrow. The fragment located close to the bone marrow grew faster than the isolated one (Text-fig. 8). The

sarcomatous fibroblasts apparently received from the ameboid cells of the bone marrow, directly or indirectly, certain substances which promoted their multiplication.

SUMMARY AND DISCUSSION.

It is an important fact that, after many months of life *in vitro*, in a medium composed exclusively of chicken plasma and embryonic juice or calf liver digest, the strain of fibroblasts isolated from Sarcoma 10 of the Crocker Foundation had kept its malignancy unimpaired. The indefinite persistence of this characteristic in a pure strain of
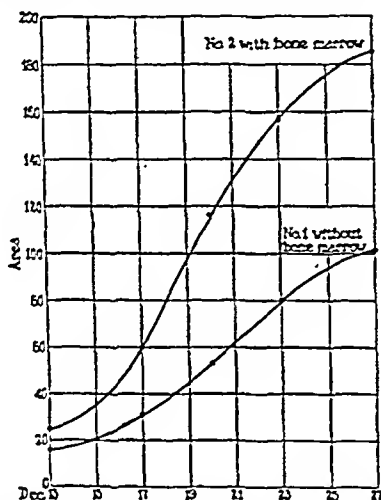
TEXT-FIG. 8.   Experiment 1518-H.   Effect of rat bone marrow on sarcomatous fibroblasts of the rat.

mammalian tissue renders possible, under ideally precise conditions, a study of the properties which are specific to malignancy.   It opens a new era in the investigation of experimental cancer of mammals. By a method similar to that here described, Fischer and Laser have isolated the active elements of Ehrlich and Flexner-Jobling carcinomas,[6] and we have recently obtained pure cultures of the Jensen sarcomatous fibroblasts.   There is little doubt that the malignant constituents of other experimental tumors can be isolated in the same

manner, and the properties associated with their unlimited growth within the body discovered.

The pure strain isolated from Sarcoma 10 is composed of fibroblasts. The cells possess all the morphological characteristics of their type. Their mode of locomotion does not differ from that of the normal fibroblasts. Their colonies are built in the same manner. However, they are larger and coarser than the normal fibroblasts of the rat when observed in pure culture (Fig. 1, *A*). Possibly, there is some other morphological difference that has not been detected by our methods of examination, such as an increase in the number of chromosomes or a change in their shape or volume. No abnormal mitoses or inclusions in the nucleus or the cytoplasm have been observed. When cultivated in a pure state, the sarcomatous fibroblasts are free from the secondary factors which *in vivo*, as well as in the mixed cultures of fresh tumor, may alter their appearance. They never degenerate and die, but multiply indefinitely. To all appearances, they are healthy cells. The malignancy that they display within the body must not be attributed to a diseased condition, but to the presence of some new physiological property.

The sarcomatous fibroblasts possess no more inherent growth energy than the normal ones. Both cell types show approximately the same residual energy in Tyrode solution and the same duration and rate of growth when placed in a nutrient medium. Like normal fibroblasts, the malignant cells multiply indefinitely in chick embryo juice, and die after a few days if cultivated in chicken or rat serum. However, they differ from the normal cells in one of their food requirements. If cultivated in a medium composed exclusively of calf liver digest, they grow in an unlimited manner, while normal fibroblasts in such a medium die within a few weeks. Although anarchical in behavior within the body, sarcomatous fibroblasts living *in vitro* require for their proliferation conditions which are almost identical with those demanded by the normal type.

However, they differ sharply from the normal fibroblasts in two aspects: they liquefy coagulated rat plasma, while normal fibroblasts do not, and they constantly produce more acid than normal fibroblasts placed under similar metabolic condition. The dissolution of the fibrin of the solid medium shows the cultures to possess the same characteris-

tics as cultures of Rous sarcoma macrophages.[11]   The coagulum assumes a striking, moth eaten appearance. The increased acid production is an expression of the phenomenon studied in the well known experiments of Warburg.[14]   If the liquefaction of the fibrin is due to its digestion, the malignant cells should be considered as setting free more active proteolytic enzymes than do normal ones. As proteoses and peptones are known to cause cell proliferation,[16] an increased peptic secretion, or an enhancement of the normal peptic activity by acid production, would explain the mechanism of unlimited growth. But the cells of Sarcoma 10 do not appear to hydrolyze serum proteins, or to obtain from them the required proteoses and peptones, since they do not proliferate in a serum medium. Therefore, it is difficult to understand how they multiply within the body more actively than normal fibroblasts.

Although interstitial lymph may be assumed to possess no more nutrient properties than diluted plasma, an attempt was made to ascertain whether it contains any growth-promoting substance for Sarcoma 10. A pure culture of sarcoma cells was grafted in the subcutaneous connective tissue of a rat for 24 hours, then removed, and cultivated in a flask. Its growth energy was found to have decreased considerably, and was recovered only after several days of life in chick embryo juice. But, when the culture was allowed to stay within the rat for 4 or 5 days, a small tumor appeared. Fragments of such a tumor cultivated in flasks immediately became surrounded with a crown of emigrated macrophages. It was obvious that the pure culture of sarcomatous fibroblasts, during its short stay in the rat, had attracted macrophages from the subcutaneous connective tissue. These macrophages are a normal constituent of the tumor *in vivo*. It may be supposed that the sarcomatous fibroblasts are supplied by the macrophages, directly or indirectly, with certain substances which are growth-promoting if acted upon by ferments. It is known that in normal tissues, macrophages may have such a nutritive function. Renaut thought that the lymph cells bring to fixed cells some food that they need.[17]   Later, it was found that macrophages effectively

[16] Carrel, A., and Baker, L. E., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 627.
[17] Renaut, J., *Arch. anat. micr.*, 1906–07, ix, 495.

promote the proliferation of normal fibroblasts.[18]   It is possible that the growth within the body of Sarcoma 10 is due to a similar phenomenon.

This hypothesis has been submitted to experimental test, and the effect of living cells on normal and sarcomatous fibroblasts cultivated in rat serum has been investigated.   When a fragment of rat bone marrow was placed between two colonies of normal and malignant fibroblasts, equidistant from both, the malignant fibroblasts proliferated far more actively than the normal ones.   At the same time, the wandering cells migrating from the bone marrow became more numerous in the acid area surrounding the sarcoma than in the normal tissue. It was obvious that, directly or indirectly, the wandering cells brought to the sarcomatous fibroblasts the substances required for multiplication.   Instead of being the expression of a defensive reaction, the macrophages of Sarcoma 10 may supply the fibroblasts with proteins such as exist in embryo juice or with protein split products which determine their unlimited proliferation within the organism. One may suppose that the growth of Sarcoma 10 depends on the simultaneous presence of two elements: the specific malignant cells, and the nursing macrophages.

CONCLUSIONS.

1. A pure strain of fibroblasts has been isolated from Sarcoma 10 of the Crocker Foundation.   After about 16 months of life *in vitro*, the malignancy of the strain is as great as that of the original tumor.

2. The strain has been compared with a strain of normal rat fibroblasts.   The malignant cells are generally larger, coarser, and more refringent than normal cells.   They possess all the morphological characteristics of fibroblasts.   They do not show any abnormalities and never degenerate and die.   They are to all appearances healthy cells.   Their mode of locomotion is identical with that of normal fibroblasts.   Their colonies are larger, but the architecture is similar.

3. The residual activity of both cell types, the duration of their life, and their rate of growth in a nutrient medium are almost identical.

4. The sarcomatous fibroblasts liquefy a rat plasma coagulum while

[18] Carrel, A., *J. Exp. Med.*, 1922, xxxvi, 385.   Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 645.   Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.

normal fibroblasts do not. They turn phenol red golden yellow whereas, under the same conditions, normal cells turn it pinkish orange.

5. Sarcomatous and normal fibroblasts of the rat multiply to an unlimited degree in chick embryo juice. They live for only a short time in rat serum and chick serum. Calf liver digest will suffice for an unlimited proliferation of sarcoma fibroblasts, but fails to support the life of normal fibroblasts for very long.

6. The presence of bone marrow greatly increases the rate of growth of sarcomatous fibroblasts cultivated in rat serum, while it only slightly affects that of the normal cells. The unlimited growth of the sarcomatous tissue in animals to which it is transplanted may be attributed to the presence of macrophages, which are a normal constituent of the tumor, and possibly are a necessary factor of its growth *in vivo*.

EXPLANATION OF PLATE 4.

Figs. 1, *A* and *B*. *A*, Culture 6827-C. Camera lucida drawing of a cell from a pure culture of fibroblasts from a normal rat. *B*, Culture 6795-C. Camera lucida drawing of a fibroblast from Sarcoma 10. Stained with 1/20,000 Janus green and 1/20,000 neutral red. The neutral red vesicles and granules are represented in gray, the fat globules by circles, and the mitochondria by lines.

Fig. 2. Culture 1482-H-1. Colonies of normal fibroblasts after 4 days in rat plasma coagulum.

Fig. 3. Culture 1482-H-2. Colony of sarcomatous fibroblasts after 4 days in rat plasma coagulum.

PLATE 4.

FIG. 1.

FIG. 2.

FIG. 3.

(Carrel and Ebeling: Properties of fibroblast and macrophage. III.)
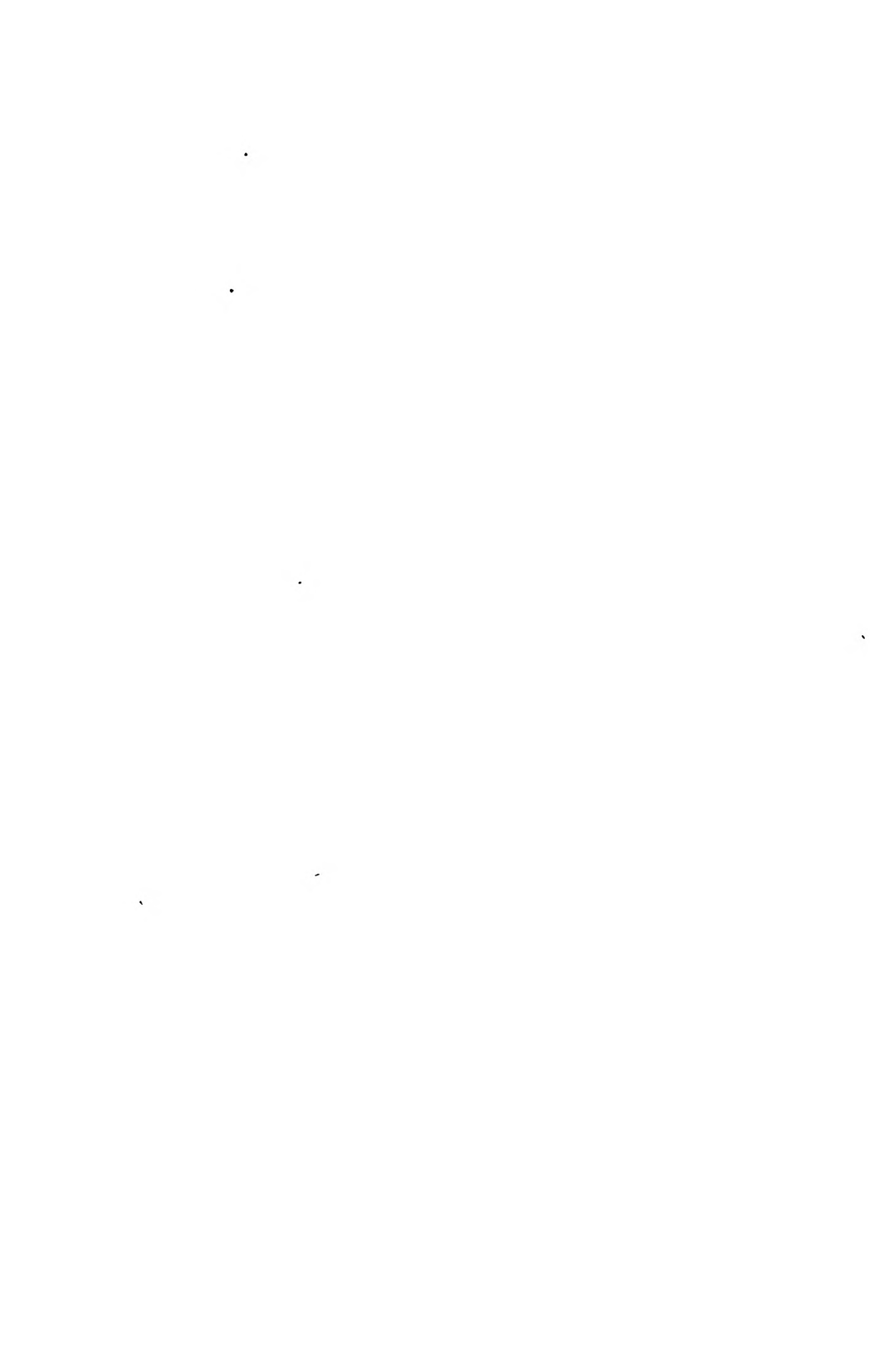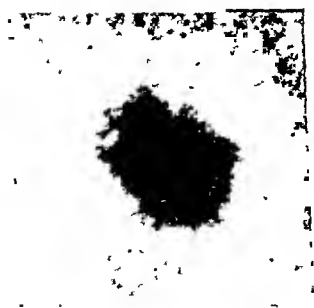
RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

II. The·Influence of Vaccinal Immunity on the Reaction to Experimental Syphilis.

By LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In the preceding paper of this series, the effects of a concomitant vaccinal infection upon the reaction to experimental syphilis in the rabbit were reported (1). It was shown that under the conditions of the experiments which involved the intracutaneous inoculation of vaccine virus at the time of an intratesticular inoculation of *Treponema pallidum*, the syphilitic reaction was profoundly disturbed in the direction of lowered efficiency, the disease which developed being much more severe than in control animals.

The syphilitic reaction has also been studied, first, in rabbits in which both vaccine virus and *T. pallidum* were inoculated simultaneously in the same site (testicle) and, second, in rabbits which had been previously immunized to vaccine virus. The study of the sort first mentioned had its origin in the unexpected results obtained with the intratesticular inoculation of syphilitic material derived from a rabbit inoculated with a strain of *T. pallidum* reported to be capable of inducing a severe infection (1). The syphilis in our inoculated animals proved unusually mild, and it was found that the original rabbit from which the strain was derived was infected as well with vaccine virus which was being transferred in the syphilitic inocula. The purpose of the second series of experiments dealing with the syphilitic reaction in vaccine-immune rabbits was to determine whether the character of the syphilis in the animals inoculated with the material containing both *T. pallidum* and vaccine virus as mentioned above, was influenced by the vaccinal immunity, an immunity well established by the time the first clinical signs of the syphilitic infection are appearing.

A preliminary report of the findings has already appeared (2). In the present paper they are described in detail.

EXPERIMENTAL.

Material and Methods.

The experiments were carried out at the same time as others previously reported (1), the same materials being used for inoculation and in like amounts. The rabbits were young adult male animals approximately 8 months of age. They were separately caged throughout the period of observation and were fed a diet of hay, oats and cabbage.

The dates of inoculation were: Experiment I, November 10, 1926; Experiment II, January 13, 1927; Experiment III, February 14, 1927.   The Nichols strain of *T. pallidum* and the Noguchi strain of vaccine virus were used, the material for inoculation in each case being obtained from actively developing testicular lesions. The emulsions prepared with the syphilitic material contained from 1 to 3 actively motile spirochetes to the microscopic field.

Control Series.—The syphilitic controls comprised 3 groups of 10 rabbits each; the same groups also served as the controls of the experiments previously reported (1).   Each animal was inoculated in 1 testicle with 0.2 cc. of the *pallidum* emulsion.

The virulence of the vaccine virus was tested in each experiment by the inoculation of 2 to 5 normal rabbits.   The inoculations (in each instance 0.2 cc. of a saline emulsion) were made intracutaneously on a shaved area of skin on the side of the body, as well as by application to a scarified skin area; an intratesticular injection was also made in certain rabbits.

Vaccine-Immune Series.—There were 3 groups of rabbits immunized to vaccine virus by the intracutaneous injection of 0.2 cc. of a virus emulsion.   Each animal developed a typical reaction, and a second injection 10 days after the first in the case of the first and second groups and 15 days later in the case of the third group, was followed by no visible reaction.   These rabbits were subsequently inoculated in 1 testicle with *T. pallidum* (0.2 cc.).   The length of time between the first injection of vaccine virus and the syphilitic inoculation was 20 days in the first, 84 days in the second and 30 days in the third experiment.   There were 5 rabbits each in the first and second groups and 10 in the third.

T. pallidum Plus Vaccine Virus Series.—There were 5 rabbits in the first and 5 in the second experiment in which a mixture of syphilitic and vaccinal emulsions was injected in 1 testicle.   In the first experiment the mixture was prepared with twice as much vaccine as syphilitic emulsion and the inoculating dose of 0.6 cc., therefore, contained 0.4 cc. of vaccinal and 0.2 cc. of syphilitic material.   In the second experiment, the mixture contained equal amounts of the 2 emulsions and each animal received 0.4 cc.

Conduct of Experiments and Analysis of Results.—The period of observation in each experiment was 3 months from the date of syphilitic inoculation.   Special attention was given to the time and frequency and to the duration of successive phases of the reaction to syphilitic infection with a view to reducing the comparison of results to as quantitative a basis as possible.   The particular conditions chosen

for comparison were: the incubation time of primary lesions, the time and frequency of the occurrence of a critical edema in the inoculated testicle, the time and frequency of occurrence of lesions in the uninoculated testicle (metastatic orchitis), the time and frequency of occurrence of generalized lesions in the skin and mucous membranes, bones or eyes, the number of foci affected by such lesions and finally, the proportion of animals that showed complete healing of all lesions during the 3 months observation period.

It should be noted, in discussing time relations of the various reactive phenomena, that there is a basic tendency toward the preservation of a uniform interval of time between the occurrence of successive reactions in syphilitic rabbits. With the Nichols strain of *T. pallidum* as carried in our laboratory, the reaction interval is approximately 2 weeks. In these experiments, as stated above, the Nichols strain was used, but the particular line from which the present substrain, as it may be termed, was derived had been transferred under somewhat different conditions from those employed with the parent strain. It was not known at the time this work was started, however, that the infection induced by this substrain would differ in any essential respect from that of the parent strain, but the results of these and other experiments have shown certain peculiar features of the syphilitic reaction, notably a delay in the development of a metastatic orchitis and an unusually early appearance of generalized lesions. These peculiarities in the behavior of the strain must be taken into account in analyzing the results obtained.

In recording the results, the term "focal distribution" or "focal incidence" as applied to generalized lesions refers to the number of discrete foci at which lesions developed as determined by actual count. The figures for actual distribution are the mean values for those animals of a group that actually developed generalized lesions, while the figures for relative distribution give the results in terms of the entire group. This distinction is made in order to permit comparison of the extent of the lesions irrespective of the number of animals affected and at the same time to avoid any erroneous impression that might arise from the chance occurrence of an occasional case of severe syphilis in any group of animals.

Results.

The results of the experiments are presented in Tables I, II and III and in Text-figs. 1 and 2.

The values in Tables I and II which represent group means, refer to the incidence and time of occurrence of various phenomena of the syphilitic reaction; Table III contains the total number of generalized lesions in the order of their appearance. The successive phases of the infection which to some extent overlap each other, are shown in Text-fig. 1 by a series of curves plotted upon a time basis. To facilitate the reading of these graphs, an arbitrary division of the curves has been made at the 35th day and those representing the incidence and the focal distribution rate of generalized lesions have been separated from the

TABLE I.

Incidence of Various Phenomena of Infection and Focal Distribution of Generalized Lesions.

Experiment	No. of rabbits	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
					Incidence	Focal distribution	Focal distribution
		per cent	*per cent*	*per cent*	*per cent*	*actual*	*relative*
I C	10	100.0	60.0	100.0	100.0	9.8	9.8
V.V. Im.	5*	100.0	100.0	100.0	75.0	8.7	6.5
V.V. (I.T.)	5	100.0		100.0	40.0	10.5	4.2
II C	10	100.0	50.0	90.0	70.0	7.0	4.9
V.V. Im.	5	100.0	40.0	80.0	80.0	4.0	3.2
V.V. (I.T.)	5	100.0		100.0	20.0	2.0	0.4
III C	10	100.0	70.0	90.0	90.0	8.4	7.6
V.V. Im.	10	100.0	40.0	90.0	90.0	7.1	6.4
Mean values							
C	30	100.0	60.0	93.3	86.7	8.6	7.4
V.V. Im.	20	100.0	55.5	90.0	89.5	6.3	5.6
V.V. (I.T.)	10	100.0		100.0	30.0	7.7	2.3

* There was 1 accidental death in this group shortly after the development of the metastatic orchitis.

In this and all other tables:

C = controls; V.V. Im. = animals immune to vaccine virus; V.V. (I.T.) = animals inoculated with *Treponema pallidum* and vaccine virus in the same testicle.

TABLE II.

Mean Time of Occurrence of Various Phenomena of Infection Estimated in Days from Date of Inoculation.

Experiment	Primary orchitis	Edema of inoculated testicle	Metastatic orchitis	Generalized lesions		
				First	Last	Mean of all
	days	*days*	*days*	*days*	*days*	*days*
I C	12.8	31.0	46.5	48.0	78.8	62.8
V.V. Im.	9.0	28.6	50.2	57.3	79.0	66.6
V.V. (I.T.)	(67.4)*		50.8	58.8	86.0	68.9
II C	22.9	40.4	61.8	62.0	80.4	67.4
V.V. Im.	20.8	32.5	56.8	63.3	73.0	68.8
V.V. (I.T.)	(44.5)*		58.2	89.0	100.0†	95.0
III C	12.2	33.9	45.3	47.6	67.6	55.0
V.V. Im.	11.0	29.3	54.3	55.3	70.4	65.9
Mean values						
C	15.8	34.7	51.0	51.6	75.1	61.0
V.V. Im.	13.0	29.5	53.7	57.2	72.6	66.6
V.V. (I.T.)	(55.9)*		54.5	68.7	90.7	71.4

* These incubation periods are enclosed in parenthesis to indicate that they are not of the same order as the others as explained in the text.

† Since only 2 generalized lesions were detected in this group, both are included in the results, although one of them developed later than the selected period of observation.

TABLE III.

Time of Appearance of Generalized Lesions Estimated from Date of Inoculation.

Days	Experiment I			Experiment II			Experiment III	
	Controls 10 rabbits	V.V. Im. 4 rabbits	V.V. (I.T.) 5 rabbits	Controls 10 rabbits	V.V. Im. 5 rabbits	V.V. (I.T.) 15 rabbits	Controls 10 rabbits	V.V. Im. 10 rabbits
35	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	1	0
39	0	0	0	0	0	0	0	0
41	1	0	0	1	0	0	2	0
43	3	0	0	0	0	0	3	0
45	0	0	0	0	0	0	4	0
47	19	0	0	0	0	0	1	1
49	3	0	0	0	0	0	9	0
51	0	0	0	0	0	0	18	2
53	6	5	0	0	0	0	12	3
55	5	1	1	0	0	0	5	5
57	11	2	0	4	1	0	0	0
59	0	0	0	7	1	0	0	6
61	7	3	6	9	2	0	0	1
63	4	0	0	7	3	0	10	9
65	6	4	1	3	1	0	1	9
67	0	0	3	4	3	0	3	0
69	1	0	0	3	0	0	0	9
71	0	0	6	1	1	0	3	10
73	0	0	0	0	2	0	1	0
75	16	7	1	1	0	0	0	0
77	8	1	1	0	0	0	3	5
79	0	1	0	0	0	0	0	3
81	0	1	1	4	2	0	0	0
83	2	0	0	0	0	0	0	0
85	0	1	0	0	0	0	0	0
87	0	0	0	2	0	0	0	0
89	1	0	1	0	0	1	0	0
91+	5	0	0	3	0	1	0	0
Total	98	26	21	49	16	2	76	63

Influence of Immunity to Vaccine Virus on Reaction to Syphilis
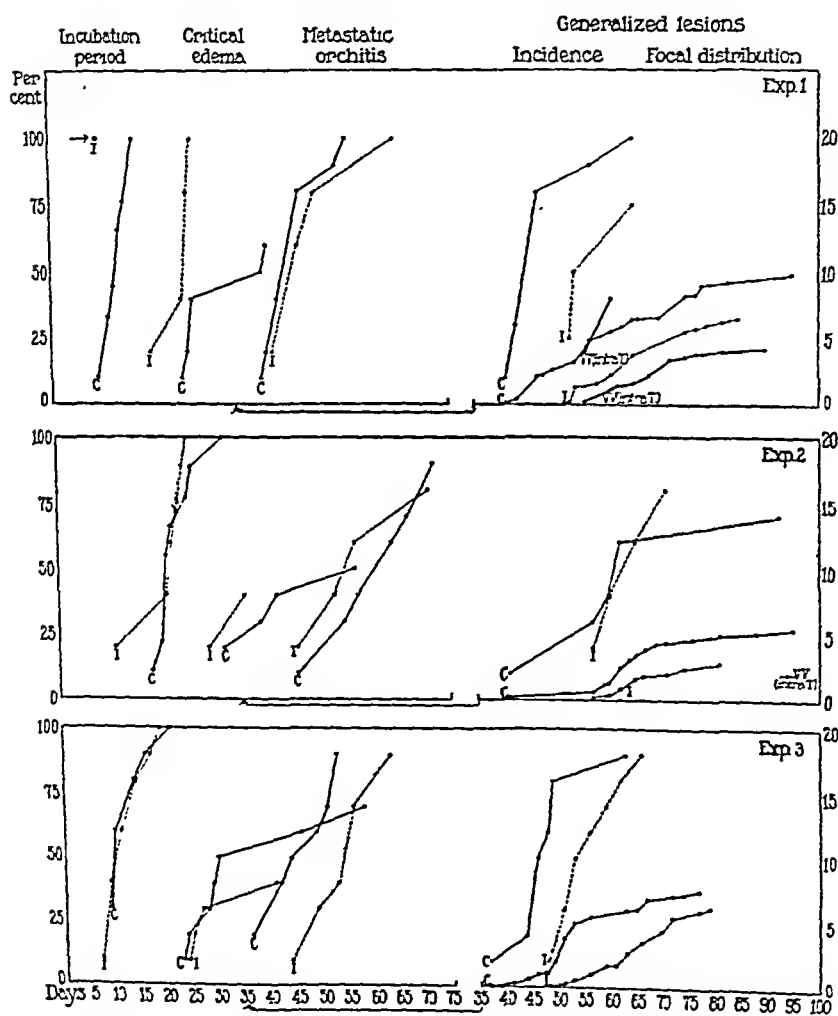
TEXT-FIG. 1.

others. The curves in Text-fig. 2 which illustrate the distribution or time of appearance of generalized lesions are compiled from the values listed in Table III.

It will be noted that whereas there were 3 groups each of vaccine-immune and control animals, there were but 2 groups of rabbits inoculated with a mixture of syphilitic and vaccinal emulsions. Strictly speaking, therefore, the results of
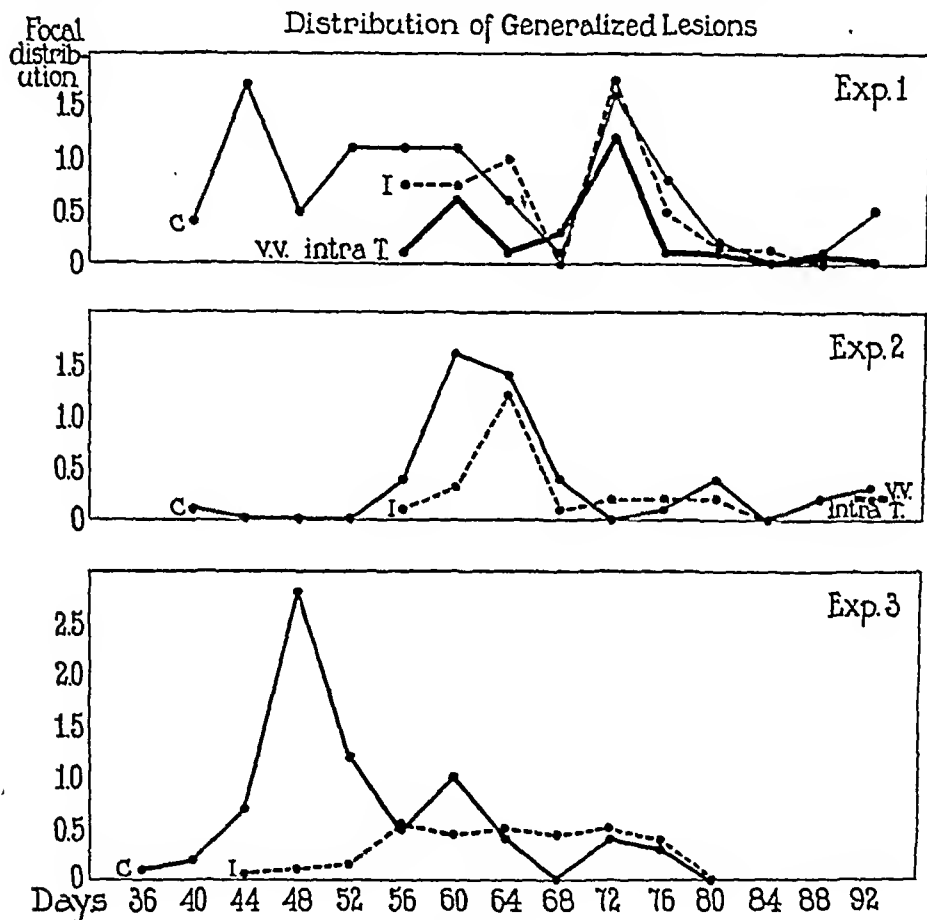
TEXT-FIG. 2.

the doubly inoculated animals should be compared with the mean values of the corresponding 2 groups of controls and vaccine-immunes, rather than with the values of the 3 groups. It has been found, however, that there is little difference between these 2 sets of mean values, so that in order to simplify the analyses of results, only the mean value of the 3 groups has been used.

DISCUSSION.

It will aid to a clearer understanding of the results obtained in these experiments if the various phenomena of the syphilitic reaction chosen for discussion are taken up in the order of their development. In this way, one obtains a picture of the infection in its successive phases and is the better able to appreciate the significance of variations in the experimental series.

Primary Orchitis.—All rabbits in each group of the 3 experiments developed a primary lesion of the inoculated testicle, that is, the controls, the vaccine-immunes and the animals inoculated intra-testicularly with a mixture of vaccinal and syphilitic viruses (Table I). The time at which the lesion was detected, however, differed in the 3 groups (Table II). The mean value of the incubation period was 15.8 days for the controls but it was slightly shorter, 13.0 days, for the vaccine-immunes. The results of individual experiments, moreover, were consistent in this respect, the difference in time being 3.8, 2.1 and 1.2 days respectively. This finding which will be discussed later in connection with other results, suggests that the character of the initial reaction of rabbits immune to vaccine virus was modified in the direction of increased susceptibility or heightened sensitivity.

In the case of the 2 groups of rabbits which were doubly inoculated, the development of a primary lesion was markedly delayed, the mean value of the incubation period being 55.9 days, a prolongation of 40.1 days beyond the mean incubation of the controls (Table II). At least 2 reasons suggest themselves as the cause of this result. In the first place, it is probable that the intense testicular reaction to vaccine virus seriously interfered with the development of spirochetes if it did not actually destroy a certain number and second, that the condition of the testicle after the subsidence of the vaccinal reaction was not such as to favor the usual growth of organisms and the production of a local lesion. In this connection, it is of interest to note that vaccine virus *per se* had no apparent immediate effect on the motility of the spirochetes as judged by successive dark-field examinations of the mixture of the 2 emulsions. Actively motile spirochetes were seen for as long as 4 hours in the mixtures kept at room temperature and in the ice box.

As will be brought out in the following discussion, the general character of the disease in any particular experiment of this nature has an important bearing on the results obtained. An idea of the variation in the disease in the 3 experiments here reported may be had by comparing the mean time at which the primary orchitis developed in the control animals. Reference to Table II shows that the incubation period in the second experiment was much longer than in the first or third, that is, 22.9 as compared with 12.8 and 12.2 days, and as will be seen, these values are indicative of the mild disease in the second and of the more severe infections in the first and third experiments. While the incubation periods of both groups of doubly inoculated animals were greatly prolonged beyond those of the controls (Table II), it was not as delayed in the second as in the first experiment, the difference in the 2 cases being 21.6 and 44.6 days respectively. This result is surprising in view of the character of the disease in the second series, for it might be thought that the effect of the vaccinal reaction upon the primary orchitis would be more marked in a mild than in a severe infection. But the dose of vaccine virus employed in the first experiment was twice as large as in the second and furthermore, the first virus was more potent than the second judging from the intracutaneous and intratesticular reactions of the control animals. It would appear, therefore, that the earlier development of the primary orchitis of the second experiment was related to a less pronounced local vaccinal reaction.

Critical Edema.—Although the phenomenon of an edema of the inoculated testicle is a variable feature of the syphilitic reaction, it is a highly significant one as it marks the end, either temporary or final, of the initial stage of the infection.

The mean incidence of edema in the control groups was 60.0 per cent as compared with 55.5 per cent for the vaccine-immune groups and with none in the doubly inoculated animals (Table I).[1] The rates for individual control groups are, on the whole, average for the Nichols strain of *T. pallidum*. The first vaccine-immune group, however, showed the high incidence of 100.0 per cent, a rate not attained by any other group in the 3 experiments. This result indicates a high

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[1] The early edema of the testicle associated with the reaction to vaccine virus is, of course, not included in the present connection.

capacity for reaction on the part of these animals, and in this connection, it should be remembered that the shortest incubation period of the primary orchitis and the greatest difference in the time of this phenomenon as compared with the controls occurred with this group. The interpretation of this finding on the basis of an initial hypersensitiveness or increased reactivity is strengthened by the uniform occurrence of a critical edema.

The time at which edema occurred shows that the vaccine-immune animals reacted more promptly than the controls. That is, in the control groups, it occurred 5 weeks after inoculation and in the vaccine-immune animals approximately a week earlier, the actual mean values being 34.7 and 29.5 days (Table II). The results of individual experiments, moreover, were consistent in this respect, the differences being 2.4, 7.9 and 4.6 days respectively.

A comparison may also be made between the time of development of the critical edema and the incubation period of the primary orchitis. The mean value of this interval, as shown in the following table, was definitely shorter in the case of the vaccine-immunes as compared with the control value. As far as individual results were concerned, the reaction interval of the vaccine-immune groups in the second and third experiments was shorter than those of the controls, while in the first experiment, it was slightly longer.

From the Incubation Period to the Critical Edema.

Experiment	Controls	Vaccine-immunes
	days	*days*
I	18.2	19.6
II	17.5	11.7
III	21.7	18.3
Mean values	19.1	16.5

The observations regarding edema of the inoculated testicle show, as was the case with the incubation period of the primary lesion, that animals which had been immunized to vaccine virus reacted more promptly to an intratesticular inoculation of *T. pallidum* than did normal rabbits.

Metastatic Orchitis.—The next phenomenon of the syphilitic reaction

to be considered is the development of a lesion in the uninoculated testicle.

As shown in Table I, the incidence of a metastatic orchitis was the same in the vaccine-immune and the control groups, the mean values being 90.0 and 93.3 per cent.   In the first experiment in which the severity of the disease was marked, all animals of both groups developed a metastatic orchitis, while in the second and third in which the disease was less severe, there was 1 rabbit each in both the control and the vaccine-immune groups in which a metastatic orchitis was never detected.   On the other hand, the incidence of a lesion in the uninoculated testicle of the rabbits which received the double inoculation of *T. pallidum* and vaccine virus was 100.0 per cent.   The fact that a metastatic orchitis developed in all doubly inoculated animals of the first experiment is not surprising in view of the severity of this particular infection.   But in the second experiment, although the disease was mild, a similar result obtained, and this finding is in harmony with the comparative shortness of the primary incubation period which was discussed above.   It is possible that the factor of individual animal variation may account for this result, and it is also possible that it is due to the variability of reaction phenomena characteristic of a mild infection.

The time of development of the metastatic orchitis as reckoned from the day of inoculation is given in Table II.   The mean value for the control groups was 51.0 days as compared with 53.7 days for the vaccine-immune animals, a difference of 2.7 days.   The differences in the first and third experiments were 4.7 and 9.0 days respectively, but in the second experiment, the order of development was reversed, the metastatic orchitis of the vaccine-immune group antedating that of the controls by 5.0 days.   Reference to the curves of Text-fig. 1 shows that in the first and third experiment, the metastatic orchitis of the controls uniformly preceded that of the vaccine-immune animals, while in the second experiment, the curve for the immunes precedes that for the controls except at one point.

The result in the second experiment is in keeping with the character of the earlier phenomena in consequence of which a restraining effect upon the subsequent phenomenon of the metastatic orchitis failed to develop.   There are at least two reasons for this behavior of the

vaccine-immune animals of this second series.   One must first take into account the known variability of reactive phenomena characteristic of a mild infection, the type which prevailed in this experiment.   In the second place, the period of vaccine immunity (84 days) was much longer than in the other experiments (20 and 30 days).   It is quite likely that if a state of initial hypersensitiveness leading eventually to a more effectual reaction, existed in the first experiment as was suggested by the observations in connection with the primary orchitis and critical edema, it might not continue over a prolonged period.[2] The curves representing the incubation period (Text-fig. 1, Experiment II) show only a slight difference in favor of an earlier development of the primary orchitis on the part of the vaccine-immune group, while the low incidence of edema makes a comparison of this phenomenon unsatisfactory, although the curve for the vaccine-immunes does precede that for the controls.   There is no clearly defined group difference in the progress of the infection up to this point as far as the time relations and the incidence of reactive phenomena are concerned.   But with the development of the metastatic orchitis, the vaccine-immune group preceded the controls, and the difference was of the same order and degree as that observed with the earlier reactions of the first experiment and resulted in a similar restraining or inhibiting effect upon later phenomena, in this case the development of generalized lesions (Text-fig. 1).   In the first experiment, the shorter incubation period of the primary lesion and the earlier development and high incidence of the critical edema in the immune animals as compared with the controls was followed by a definite delay in the development of the next phenomena, namely, the metastatic orchitis and the generalized lesions.   In the third experiment, group differences in the time relations of the primary orchitis and critical edema are not so clear-cut as in the first experi-

[2] It should also be noted that the rabbits of the second vaccine immune groups had been living under laboratory conditions approximately 3 months longer than the controls.   This factor of caging and of age as well, undoubtedly had some influence upon the character of the reaction, but it is difficult of estimation, for while its effect is usually seen in the direction of increased efficiency, or in what may be termed a lower initial reactive state, such effects are not invariable or constant.

ment, but they become well marked with the development of the metastatic orchitis and again with the time of appearance of the first generalized lesions, as shown by the curves of Text-fig. 1.

The development of a metastatic orchitis in the doubly inoculated animals occurred in 50.8 and 58.2 days after inoculation (Table II). As compared with the controls, it was delayed 4.6 days in the first experiment, but in the second it preceded the control value by 3.6 days. The small magnitude of these values is of especial interest in that a greater delay in the development of a metastatic orchitis might be expected in view of the profound disturbance of the primary orchitis caused by the early vaccinal reaction. In the first experiment, the lesion of the uninoculated testicle developed 16.6 days earlier than that of the inoculated testicle, while in the second, the results were reversed, the primary orchitis developing 13.7 days before the metastatic lesion. But it should be noted that despite these differences, the development of the metastatic orchitis in both experiments occurred in comparable periods of time and curiously enough there was less difference between them (8.4 days) than between the corresponding control values (15.3 days).

Generalized Lesions.—The phenomenon of generalized lesions is the most significant single basis for estimating the general character of the syphilitic reaction. The occurrence or non-occurrence of generalized manifestations, the time of their appearance, their number, nature and persistence are among the features of this phase of the infection which may be used in evaluating its character.

The mean incidence of generalized lesions was practically the same in the control and vaccine-immune groups as shown in Table I and there were no significant differences in individual experiments except in the first. Here the disease was very severe and all controls developed secondary manifestations, but there was one vaccine-immune animal in which none was detected. In the doubly inoculated rabbits, on the other hand, there was a low incidence of generalized lesions, the mean value of 30.0 per cent contrasting with 86.7 per cent for the controls (Table I).

In regard to the distribution of generalized lesions, the mean values given in Table I show definite group differences. The following

analysis gives the extent of these differences in percentage terms of the control values:

Group	Actual rate	Relative rate
	per cent of control value	*per cent of control value*
Controls......................................	8.6	7.4
Vaccine-immunes..............................	6.3 or −26.72	5.6 or −24.32
V.V. (I.T.)....................................	7.7 or −10.46	2.3 or −68.92

As has been stated in the section on Material and Methods, the relative rate of focal distribution which is estimated upon the total number of animals in the group, gives a more comprehensive idea than the actual rate of the extent of the lesions irrespective of the number of animals affected. This point is illustrated by the actual and relative rates for the doubly inoculated animals as shown in the above table. The magnitude of the actual rate is disproportionately large, due to 2 cases of moderately severe syphilis and the relative rate is a fairer expression of the group as a whole. From the figures given above, it is clear that as far as the distribution of generalized lesions is concerned, the infection of the doubly inoculated animals was much less pronounced than that of the controls, and while in the vaccine-immunes it was not as mild as in the doubly inoculated rabbits, still it was definitely less severe than in the controls.

In regard to the initiation of this phase of the reaction as determined clinically, the mean values as given in Table II show that generalized lesions appeared first among the controls and last among the doubly inoculated animals. The difference in time as compared with the control value was 5.6 and 17.1 days for the vaccine-immune and doubly inoculated rabbits, respectively, and as far as individual experiments were concerned, the results for both groups were consistent. In the first and third experiments in which the disease was severe, the delay in the appearance of generalized lesions in the vaccine-immunes as compared with the controls amounted to 9.3 and 7.7 days respectively. The delay in the second experiment was only 1.3 days, a negligible difference from the standpoint of magnitude, but of some importance in that it conforms to the results of the other experiments. That a greater difference did not occur is probably due

to the mild character of the disease.   In the case of the doubly inoculated animals of the first experiment, generalized lesions first appeared 10.8 days after the controls while in the second series, their appearance was delayed 27.0 days beyond the control group.   The difference in the character of the disease probably explains the difference in the magnitude of these results, that is to say, the effect induced by the double inoculation in the direction of delayed reaction was to some extent offset in the first experiment by the severity of the infection.

With regard to the last generalized lesions to appear, as shown in Table II and Text-fig. 1, there was little difference between the vaccine-immune and control animals.   But with the doubly inoculated rabbits, the development of the few generalized lesions which did appear was so delayed that the mean value for the last lesion is considerably larger than the control figure, that is, 90.7 and 75.1 days respectively.

Further differences in the reaction of the vaccine-immune groups as compared with the controls are brought out in the matter of the time relationships existing between the initiation of the phenomenon of generalized lesions and those which preceded it as shown in the following table:

From the Primary Orchitis to the First Generalized Lesions.

Group	Experiment I	Experiment II	Experiment III	Mean values
	days	days	days	days
Controls.....................................	35.2	39.1	35.4	35.8
Vaccine-immunes........................	48.3	42.5	44.3	44.2

From the Critical Edema to the First Generalized Lesions.

Controls.....................................	17.0	21.6	13.7	16.9
Vaccine-immunes........................	28.7	30.8	26.0	27.7

From the Metastatic Orchitis to the First Generalized Lesions.

Controls.....................................	1.5	0.2	2.3	0.6
Vaccine-immunes........................	7.1	6.5	1.0	3.5

Considering only the mean values given above, the reaction intervals of the vaccine-immune animals exceeded those of the controls to the following extent:

per cent

From the primary orchitis to the first generalized lesion......... 23.5
From the critical edema to the first generalized lesion........... 63.9
From the metastatic orchitis to the first generalized lesion....... 48.3

It has already been pointed out that a critical edema is a variable phenomenon and this fact together with the difference in the number of animals composing the 2 groups, contributes to the magnitude of the value resulting from the comparison of the critical edema and generalized lesion interval. But the above analyses show very clearly that the reaction of the vaccine-immune rabbits in the case of the generalized manifestations of disease was not as prompt as that of the control animals. Similar comparisons with the doubly inoculated rabbits are not applicable because of the irregularities already noted in connection with the early phenomena of the infection.

A significant aspect of the reaction to syphilis is the duration of the period in which generalized manifestations develop. In the following table the controls and the vaccine-immune animals are compared from this standpoint, using the values in Table II for the time of appearance of the first and last generalized lesions, and it is at once apparent that this phase of the reaction was considerably shortened in the case of the vaccine-immune groups.

Duration of Active Period of Generalized Lesions.

Experiment	Controls	Vaccine-immunes	Difference in time	Per cent of control values
	days	*days*	*days*	*per cent*
I	30.8	21.7	−9.3 or 30.2	
II	18.4	10.0	−8.0 or 43.5	
III	20.0	14.8	−5.2 or 26.0	
Mean values	23.5	15.5	−8.5 or 36.2	

In regard to the doubly inoculated animals, the active period of generalized manifestations in the first experiment was 27.5 days as compared with 30.8 days for the controls (Table II). It will be remembered, however, that in this experiment the incidence of generalized lesions was 40.0 and 100.0 per cent for the doubly inoculated and control groups respectively (Table I) and although this period of eruptive activity was but slightly shortened in the case of the

doubly inoculated groups, the compared value represents only 2 animals. In the second experiment only 1 doubly inoculated rabbit (20.0 per cent) developed 2 generalized lesions separated by an interval of 11 days as compared with an incidence of 70.0 per cent among the controls and a period of active development of 18.4 days (Tables I, II and III). It would appear, therefore, that there was a definite tendency on the part of the doubly inoculated rabbits toward a shortening of the active period of generalized manifestations as was the case with the vaccine-immune rabbits.

The mean time of appearance of *all* generalized lesions together with their time distribution may also be used as a basis of comparing the syphilitic reaction. The majority of lesions in the vaccine-immune and doubly inoculated animals tended to develop later than in the controls, as indicated by the mean values given in Table II, that is, 66.6 and 71.4 days as compared with the control figure of 61.0 days, but the difference is more strikingly brought out in the distribution curves of Text-fig. 2. Since the relative focal distribution rates have been employed in making these curves, their height gives an idea of disease severity. The delay in the initiation of secondary lesions, the generally lower level of the curves and the tendency for the majority of lesions to develop late in the course of the disease are uniform features of the reaction in the vaccine-immunes and doubly inoculated animals not observed in the controls.

Resolution and Healing of Lesions.—The last phase of the reaction to be considered is the state of the lesions at the conclusion of the experiment, that is, 3 months after inoculation. By this time, in a group of 5 or 10 rabbits and under ordinary conditions of intratesticular inoculation with a virulent strain of *T. pallidum*, a certain number of animals still show active lesions; in others, the lesions are regressing, while in the remainder, the manifestations of the disease are healed. A comparison of experimental groups from this standpoint furnishes the basis, therefore, for estimating the character of the syphilitic reaction in its capacity toward the accomplishment of a latent state. The following table summarizes the observations on the state of the lesions in the various groups of the present experiments 3 months after inoculation.

Final State of the Lesions.

Experiment	Healed		Not healed		Active	
	No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
I C	1	10.0	5	50.0	4	40.0
V.V. Im.	1	25.0	2	50.0	1	25.0
V.V. (I.T.)	1	20.0	2	40.0	2	40.0
II C	0		6	60.0	4	40.0
V.V. Im.	3	60.0	2	40.0	0	
V.V. (I.T.)	3	60.0	2	40.0	0	
III C	0		10	100.0	0	
V.V. Im.	2	20.0	8	80.0	0	
Mean values						
C	1	3.3	21	70.0	8	26.7
V.V Im.	6	31.6	12	63.2	1	5.3
V.V. (I.T.)	4	40.0	4	40.0	2	20.0

This analysis shows that in the late stage of the disease the activity of the syphilitic process as measured by the state of the lesions was much less pronounced in the vaccine-immunes than in the controls as shown by contrasting the percentage of animals in which the lesions either were healed or were still active. In the case of the doubly inoculated rabbits, a similar result obtained as regards the proportion of animals with healed lesions but not with respect to those which still showed active manifestations. This variant result was due to 2 doubly inoculated rabbits in the first experiment in which the disease was of average severity as far as the focal distribution of generalized lesions was concerned, although their appearance was delayed beyond those of the controls (Tables II and III; Text-fig. 1), so that active manifestations were present in these animals 3 months after inoculation. And it should be emphasized that these 2 rabbits were the only ones in either the first or second groups of doubly inoculated animals which developed a disease of even average severity; in all others it was extremely mild. If the mean values of the above table are combined on the basis of the presence or absence of syphilitic lesions, irrespective of degree of activity, a distinct difference between the vaccine-immunes and doubly inoculated animals on the one hand, and the controls on the other, is again brought out.

Group	Lesions healed	Lesions present
	per cent	*per cent*
Controls..	3.3	96.7
Vaccine-immunes....................................	31.6	68.5
V.V (I.T.)..	40.0	60.0

From the foregoing discussion of the reaction to syphilis in rabbits immunized to vaccine virus and in rabbits inoculated intratesticularly with *T. pallidum* and vaccine virus,.it is clear that the reaction of both sets of animals differed from that of the controls. As far as the vaccine-immune rabbits were concerned, the time relations of the early phenomena, that is, the primary lesion and the critical edema, were shorter than in the control groups, suggesting an initial condition of hypersensitiveness. But the subsequent manifestations of a metastatic orchitis and of generalized lesions developed later than in the controls, and in addition, the distribution rates of generalized lesions were lower, the period of activity of this phase of the infection was much shorter, and in a higher proportion of animals, all lesions had become healed 3 months after inoculation. From the standpoint of the entire disease picture, therefore, it is evident that the effectiveness of the host's reaction was increased by the presence of vaccine immunity at the time of inoculation with syphilis.

With respect to the animals inoculated simultaneously in the testicle with *T. pallidum* and vaccine virus, the results on the whole conform to those obtained in the vaccine-immune rabbits as far as the later phases of the syphilitic reaction were concerned, including the important one of generalized lesions. The results connected with the early syphilitic phenomena were of an entirely different order from those which obtained in the vaccine-immunes and in the controls due, presumably, to the local effects of the vaccinal reaction. The modification of the syphilitic reaction in the doubly inoculated animals in the direction of increased effectiveness has been interpreted as due, in part at least, to the effect of a vaccinal immunity. It is known that vaccine immunity in rabbits is well developed 2 to 3 weeks after inoculation of the virus and continues for an indefinite time. The influence of such an immune state upon the syphilitic reaction would, therefore, be operative during the period of development of syphilitic

phenomena, and the present experiments show that the modification of the syphilitic reaction was of the same general order as that observed in rabbits immune to vaccine virus at the time of *pallidum* inoculation.

Mention was made at the beginning of this paper that the results obtained with a certain strain of *T. pallidum* were entirely unexpected in that the strain is reported to be capable of producing a severe disease and that which developed in our rabbits was extremely mild. It was found that the original rabbit from which we obtained the strain was also infected with vaccine virus and furthermore, that the virus was being transferred with the syphilitic inocula. In the light of the experiments reported in this paper, the reason for these unexpected results is now clear, namely, a more effective reaction to the syphilitic infection associated with the development of vaccinal immunity. To what extent the results were also influenced by the local reaction from the contaminating vaccine virus is difficult to say, but it is certain that the vaccinal reactions were not as marked in the experiments referred to above as those in the experiments described in the present paper.

The results reported in this paper are entirely different from those obtained in other experiments in which vaccine virus and *T. pallidum* were also inoculated simultaneously but in different sites, that is, vaccine virus intracutaneously on the side of the body and *T. pallidum* intratesticularly (1). Under these conditions of inoculation, the effectiveness of the syphilitic reaction was greatly decreased, resulting in a severe infection, and this despite the development of an intense vaccinal reaction and a subsequent vaccinal immunity. It is evident, therefore, that the manner in which two concomitant experimental infections are induced is an important factor in determining the nature and capacity of the host's reaction to one of them.

SUMMARY.

Experiments are reported in which it was shown, first, that the effectiveness of the reaction to experimental syphilis was increased in rabbits immune to vaccine virus, the ensuing disease being less severe than in control animals.

It was further shown that a comparable modification of the syphilitic reaction occurred in rabbits inoculated intratesticularly with a mixture of *Treponema pallidum* and vaccine virus, and it was suggested that this result was due to the influence of a vaccinal immunity and to the local effect of the vaccinal reaction.

It was pointed out that the manner in which two concomitant experimental infections are induced is of significance in determining the character of the host's reaction.

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THE INCUBATION PERIOD OF YELLOW FEVER IN THE MOSQUITO.

By JOHANNES H. BAUER, M.D., and N. PAUL HUDSON, M.D.

*(From the Laboratories of the Commission on Yellow Fever, International Health
Division of the Rockefeller Foundation, Lagos, Nigeria, West Africa.)*

During the yellow fever epidemic at Orwood and Taylor, Mississippi,
in 1898, Carter (1, 2) noted that an interval of from 2 to 3 weeks
elapsed between the first infecting cases and the secondary cases
arising therefrom. This observation antedates, of course, the demon-
strations of mosquito transmission of the disease. In 1900 Reed,
Carroll, Agramonte and Lazear (3) in establishing the fact of mosquito
transmission discovered that a period of about 12 days after biting was
required before the mosquito was capable of inducing the infection in
normal individuals. They showed, for example, in one experiment,
that several mosquitoes which had fed on yellow fever patients, were
non-infective on the 4th and 7th days and infective to the same
individual on the 17th day following the original feeding. Guiteras
(4) confirmed this finding. Mosquitoes having fed on a yellow fever
patient were non-infective to a susceptible person on the 5th and infec-
tive on the 20th day after the feeding. Marchoux, Salimbeni and
Simond (5) who made numerous such tests concluded that at least 12
days must elapse between the feeding of mosquitoes (*Aedes ægypti*)
on yellow fever patients and their ability to transmit the infection
to fresh susceptible persons. This period between the initial infect-
ing feeding and the time at which the mosquitoes are capable of induc-
ing infection explains and corresponds to what has been called by
Carter "extrinsic incubation."

Now that it has been shown (6) that the virus of yellow fever is
transmissible to *Macacus rhesus*, in which species of monkey an experi-
mental disease is produced corresponding to yellow fever in man, it
becomes possible to determine more precisely the exact period of this
extrinsic incubation. Earlier experiments (6) had shown that whereas

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the virus is readily filtrable when in the circulating blood, the filtrates prepared from mosquitoes which had fed on infected animals were ineffective on inoculation. The experiments to follow were designed for the purpose of elucidating the different states in which the virus is supposed to exist in the mosquito during extrinsic incubation and in man and monkey during actual infection. A uniform number of mosquitoes were made to bite normal *Macacus rhesus* monkeys at various intervals after the infectious feed, after which they were ground up and injected into susceptible monkeys.

The temperature in the storage cage in which the mosquitoes were kept during these experiments was registered with a recording thermometer and was found to vary daily between 74° and 90°F.; the coolest time was between 6 and 8 o'clock in the morning and the warmest between 3 and 5 in the afternoon.

Experiment 1.—On January 28, 1928, a normal *rhesus*, No. 50–6, was exposed to Lot 27 of infected *A. ægypti*, eighteen of which fed upon this animal. On February 3 the monkey showed a temperature of 105.8°F., and on that date Lot 35, consisting of 95 normal females, *Aëdes ægypti*, were made to feed on the animal.

At intervals of 48 hours, five mosquitoes of this lot were transferred to a suitable cage and a normal monkey exposed until each of these five insects had fed. The mosquitoes were then caught, killed with tobacco smoke, ground up in a mortar with 3.0 cc. of salt solution and the emulsion injected subcutaneously into another normal *rhesus* monkey. This procedure was carried out 2, 4, 6, 8, 10 and 12 days after the original feeding of the mosquitoes on *Rhesus* 50–6. The mosquitoes of this lot died very rapidly so that on February 15, *i.e.*, on the 12th day after the original feeding, only three remained alive, which were used instead of five.

As seen from Table I, the results of this experiment were irregular. None of the mosquitoes which fed on normal monkeys 2, 4, 6, 8 and 10 days after the original infectious feed induced disease, while those tested on the 12th day produced infection. On the other hand, 2, 4 and 8 day mosquitoes, which failed to infect by biting, proved infectious when used for injection into monkeys. 12 day mosquitoes produced infection by both methods of inoculation, while 6 and 10 day insects failed by biting and by injection. All monkeys failing to respond were shown to be susceptible by subsequent inoculation with virulent blood or by biting with known infected mosquitoes.

The length of life of a female *A. ægypti*, under laboratory conditions,

irrespective of whether the insect is infected with yellow fever virus or not, averaged in our experience about 2 months, and about 10 per cent of the insects usually lived over 3 months. Occasionally, however, some batches of mosquitoes became subject to epidemics in which all insects that happened to be in one cage died within a few days, irrespective of their age, food, surrounding temperature, humidity, etc.

TABLE I.

Experiment 1. Mosquitoes, Lot 35, Infected February 3, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 5	2	51-1	5 mosquitoes fed	No reaction
" 5	2	51-2	Same mosquitoes macerated and injected	Feb. 17. Died. Postmortem findings typical
" 7	4	51-3	5 mosquitoes fed	No reaction
" 7	4	51-4	Same mosquitoes macerated and injected	Feb. 13. Died. Postmortem findings typical
" 9	6	51-5	5 mosquitoes fed	No reaction
" 9	6	51-6	Same mosquitoes macerated and injected	No reaction
" 11	8	51-7	5 mosquitoes fed	No reaction
" 11	8	51-8	Same mosquitoes macerated and injected	Feb. 17. Died. Postmortem findings typical
" 13	10	51-9	5 mosquitoes fed	No reaction
" 13	10	52-0	Same mosquitoes macerated and injected	No reaction
" 15	12	52-1	3 mosquitoes fed	Feb. 26. Died. Postmortem findings typical
" 15	12	52-2	Same mosquitoes macerated and injected	Feb. 24. Died. Postmortem findings typical

According to Howard, Dyar and Knab (7), these epidemics have been studied by numerous workers and have been attributed mostly to fungi and occasionally to bacteria which are supposed to invade the digestive tract and the body cavities. Having no other explanation, it seems to us that the high mortality in the mosquitoes of Lot 35 was due to one of these parasitic infections. If this was the case, it seems also possible that the yellow fever virus might in the 6 and 10 day mosquitoes have been destroyed by the invading organisms. This

hypothesis seems to be supported by the results obtained in subsequent experiments.

Experiment 2.—Rhesus 51–8, which in the above experiment on February 11 had been inoculated subcutaneously with the emulsion of five macerated mosquitoes, continued to show normal temperature until February 15, when the temperature suddenly rose to 105.3°F. The monkey was immediately exposed to a lot of

TABLE II.

Experiment 2. Mosquitoes, Lot 36, Infected February 15, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 18	3	54-5	10 mosquitoes fed	No reaction
" 18	3	54-6	Same mosquitoes macerated and injected	Feb. 27. Died. Postmortem findings typical
" 20	5	54-7	10 mosquitoes fed	No reaction
" 20	5	54-8	Same mosquitoes macerated and injected	Feb. 27. Died. Postmortem findings typical
" 22	7	54-9	10 mosquitoes fed	No reaction
" 22	7	55-0	Same mosquitoes macerated and injected	Mar. 1. Died. Postmortem findings typical
" 24	9	55-1	10 mosquitoes fed	Mar 2. Died. Postmortem findings typical
" 24	9	55-2	Same mosquitoes macerated and injected	Feb. 29. Died. Postmortem findings typical
" 26	11	55-3	10 mosquitoes fed	Mar. 2. Died. Postmortem findings typical
" 26	11	55-4	Same mosquitoes macerated and injected	Mar. 6. Died. Postmortem findings typical
" 28	13	55-5	10 mosquitoes fed	Mar. 5-9. Fever. Recovered
" 28	13	55-6	Same mosquitoes macerated and injected	Mar. 8. Died. Postmortem findings typical

normal *A. ægypti*, of which 130 became engorged. These were labelled "Lot 36" and were used in the next experiment.

Beginning on the 3rd day after feeding on *Rhesus* 51–8, and thereafter on every other day, ten mosquitoes were transferred to a suitable cage and fed on a normal monkey. After the monkey was removed, the engorged mosquitoes were caught, killed with tobacco smoke, ground up in a mortar with 3.0 cc. of salt solution and the emulsion injected subcutaneously into another normal monkey. This procedure was carried out on the 3rd, 5th, 7th, 9th, 11th and 13th days from the date when the mosquitoes had originally fed on the infected monkey. No mortality

occurred among the mosquitoes of this lot to interfere with the completion of the experiment.

The results are summarized in Table II. This experiment shows that the virus is definitely infectious during the entire period of the extrinsic incubation. Typical yellow fever was produced in monkeys by injecting the substance of the mosquitoes at any time after the insects had ingested the virus from an infected animal. Although then present in the mosquitoes in an infectious form, the virus was not, however, transmitted by bite until from the 9th day on. As seen from this table, all the animals which developed infection, either as a result of the injection of mosquito emulsion or through bite of the infected insects, died with the exception of one, *Rhesus* 55–5, which had been bitten by the 13 day mosquitoes. 5 days after the bite this monkey developed high temperature which continued for another 5 days. Final recovery ensued and upon subsequent inoculation with a large dose of virulent blood, the animal again ran a febrile period but did not succumb. The three monkeys which were bitten by 3, 5 and 7 day mosquitoes and did not respond, were found as susceptible as normal animals to later inoculation with virulent blood.

In order to confirm these findings, one more experiment was carried out, with the same technique and the same number of mosquitoes, but over the time intervals at which Experiment 1 was performed.

Experiment 3.—*Rhesus* 51–3, which in Experiment 1 had been bitten by 4 day mosquitoes without response, was on February 20 exposed to two infected mosquitoes, of which one only fed. On the afternoon of February 23, the animal's temperature was 106.0°F.; it was then exposed to a lot of normal *A. ægypti* and 102 of these became engorged. These, labelled "Lot 42," were used in the following experiment.

Beginning on the 4th day after feeding on *Rhesus* 51–3, and thereafter on every other day, ten of these mosquitoes were placed in a suitable cage and made to feed on a normal monkey. After the monkey was removed, the engorged mosquitoes were caught, killed with tobacco smoke and ground up in a mortar with 3.0 cc. of salt solution. The emulsion was injected subcutaneously into another normal monkey.

The results are given in Table III. All monkeys which had been inoculated with macerated mosquito emulsions at various intervals after the insects had their infectious feed, died of typical yellow fever infection. Those, on the other hand, which had been bitten by the

same mosquitoes at the same intervals, gave no response with the exception of one monkey, No. 56–5, on which 12 day mosquitoes were allowed to feed, and which died of typical yellow fever. Monkeys 55–7, 55–9 and 56–1 were later either inoculated with virulent blood or exposed to mosquitoes which were known to be infected and died of yellow fever, thus proving their susceptibility. However, Monkey 56–3, which was bitten by 10 day mosquitoes and showed no reaction,

TABLE III.

Experiment 3. Mosquitoes, Lot 42, Infected February 23, 1928.

Date of experiments	No. of days after original feeding	No. of monkey	Mode of transmission	Results
Feb. 27	4	55-7	10 mosquitoes fed	No reaction
" 27	4	55-8	Same mosquitoes macerated and injected	Mar. 10. Died. Postmortem findings typical
" 29	6	55-9	10 mosquitoes fed	No reaction
" 29	6	56-0	Same mosquitoes macerated and injected	Mar. 8. Died. Postmortem findings typical
Mar. 2	8	56-1	10 mosquitoes fed	No reaction
" 2	8	56-2	Same mosquitoes macerated and injected	Mar. 11. Died. Postmortem findings typical
" 4	10	56-3	10 mosquitoes fed	No reaction; later proved immune
" 4	10	56-4	Same mosquitoes macerated and injected	Mar 16. Died. Postmortem findings typical
" 6	12	56-5	10 mosquitoes fed	Mar. 16. Died. Postmortem findings typical
" 6	12	56-6	Same mosquitoes macerated and injected	Mar. 12. Died. Postmortem findings typical

proved refractory when given 2 weeks later a large dose of virulent blood. It is impossible to determine whether the last animal was naturally immune or had acquired immunity as the result of the mosquito bite.

SUMMARY.

1. The yellow fever virus was found in infectious form in *Aedes ægypti* throughout the entire period of the extrinsic incubation, as demonstrated by the injection of the bodies of mosquitoes into normal

rhesus monkeys at daily intervals after the insects had fed on an infected animal.

2. The virus was transmitted through the bite of the mosquitoes, in one experiment on and after the 9th day, and in two experiments on the 12th day after the initial infecting feed.

3. The pathologic changes produced by the injection of the infected mosquitoes into normal monkeys during the extrinsic incubation were in every respect those of typical experimental yellow fever.

4. The monkeys withstand easily the subcutaneous injection of the mosquito emulsion.   No acute inflammatory reaction was observed at the site of injection in any of the seventeen animals inoculated with this material in these three experiments.

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OF

EXPERIMENTAL MEDICINE

THE FATE OF HUMAN AND BOVINE TUBERCLE BACILLI IN VARIOUS ORGANS OF THE RABBIT.

By MAX B. LURIE, M.D.

(*From the Henry Phipps Institute, University of Pennsylvania, Philadelphia.*)

In a recent study of the susceptibility of various organs of the rabbit to tuberculosis (1) attention was drawn to the fact that their "native" cellular reaction to the intravenous injection of heat-killed tubercle bacilli differed greatly. From this and from other data the inference was drawn that different organs in a given species possess an inherently different power to destroy tubercle bacilli before any allergic manifestations are apparent. It was thought desirable to examine this problem more thoroughly.

Now, while dead bacilli elicit the cellular reaction to the chemical constituents of the organism, they do not reproduce all the conditions of infection with living bacilli. Dr. Eugene L. Opie suggested that the problem be attacked with living bacilli by cultural methods. Obviously this approach complicates the problem, but the results so obtained are more significant.

Cultural methods have often been used for the study of infectious diseases and for the isolation of tubercle bacilli from various sources, but the only instance of their use we know of, for the quantitative estimation of tubercle bacilli in a given source, is Lange's attempt (2) to estimate the number of living bacilli by the number of colonies that developed from a given suspension of tubercle bacilli planted on serum egg medium. After a few trials with (3) Boissevain's plasma method, which was found to be unsuitable for our purpose, preliminary experiments were performed with Dorset's egg medium and Petroff's gentian violet medium. It soon appeared that, while the method does not give the absolute number of living tubercle bacilli in a given specimen of tissue, it gives the relative numbers with sufficient accuracy. During the progress of this work Madsen and Mørch (4) have reported

a close correspondence between Petroff's method and guinea pig inoculation in their studies on rabbits treated with sanocrysin.

Method.

At varying intervals of time after the intravenous injection of varying quantities of different types of tubercle bacilli, the rabbit is killed by a blow on the head. The hair on the entire ventral surface of the animal is removed by a depilatory mixture and the skin washed and covered with an antiseptic. With rigorous precautions for sterility, the skin is reflected. The abdominal cavity is opened, a separate pair of sterile instruments being used for each step. The first organ that presents itself in the wound is the liver. 1 gm. quantity is weighed accurately into a sterile Petri dish. In a similar manner like weighed amounts of spleen and kidney are excised as they are successively uncovered. The thoracic cavity is now opened and 1 gm. quantity of lung, representative in its gross pathological condition of the entire organ, is removed. Aseptically, the right femur is crushed with forceps and a weighed amount, as near a gm. as possible, of bone marrow is removed. The specimens of tissue are then ground in sterilized mortars with sterilized sand as finely as possible consistent with sterility and are suspended in a given quantity of fifteenth molar disodium phosphate (5). From these suspensions, containing a known weight of a given tissue, dilutions are made in the same alkaline medium. 1 cc. quantity of each dilution of each organ is shaken in a sterilized tube containing glass beads and seeded directly on three tubes of Dorset's medium and three tubes of Petroff's medium, divided as equally as possible and spread as evenly as possible on the surface of the medium by means of a pipette. The suspended material is allowed to settle for at least ½ hour, often much longer. Uniform distribution of the inoculated material is secured by allowing the tubes to recline so that the surface of the medium is in a horizontal plane. The tubes are now incubated. Another 1 cc. specimen is treated with 3 per cent sodium hydroxide according to Petroff's method, centrifugalized, neutralized with hydrochloric acid, and seeded on three tubes of Petroff's and Dorset's medium respectively as before. After the tubes have been left in the incubator for varying periods of time, the number of tubercle bacillus colonies in each is counted and recorded at weekly or biweekly intervals. Repeated readings are necessary both to check the counts and also to prevent loss of any data that might result from subsequent contamination.

We found that at least 3 months must elapse before the ultimate number of colonies in any one tube could be determined, for often new colonies formed even as late as 10 weeks after incubation, especially with the bovine strain. Since the tubes must be maintained at incubator temperature for such a long time all precautions against drying out of the medium are necessary. Precautions must also be taken against the spreading of colonies by the liquid of condensation. For these reasons, the tubes were always read in an upright position and handled as

little as possible. When the number of colonies appearing on the surface of a tube was not more than 200 they could easily be counted directly. When the colonies exceeded this number, they were estimated by wrapping about the tube a piece of x-ray celluloid from which square windows of varying sizes had been cut out and reading these areas with a magnifying glass. Repeated readings tended to eliminate gross errors. In case of any doubt whether a given colony was of tubercle bacilli or not, smears were made and stained by the Ziehl-Neelsen method.

Strains.—Two human strains and one bovine strain were used. The human strains, P-15 B and P-48 A, were isolated in the latter part of 1923 from a nodule in the lung and from a hilum lymph node respectively of human autopsy material (6). Both were typical human strains as determined by rabbit inoculation. The bovine strain, Bovine C, was also isolated in 1923 by Dr. Joseph D. Aronson from a lymph node of a tuberculous cow. Originally it was exceptionally virulent for the rabbit (7) and it still behaves like a typical virulent bovine strain.

Dosage.—In one series of experiments the fate of the human tubercle bacillus, P-15 B, was compared with the fate of the bovine type, Bovine C, in the various organs of the rabbit in quantities of 0.1 mg. each to rabbits of approximately the same weight. In another series the behavior of the same bovine strain was compared with another human strain, P-48 A, in quantities of 0.001 mg. per kilo of body weight.

The animals were killed 2 days, 1, 2 and 4 weeks and 2 months after intravenous infection and at other intervals, as will be apparent from the tables and curves.

The figures in the tables represent the average number of colonies recovered, usually from three separate tubes, each planted with a like quantity of the same suspension of tissue of a given dilution. When two dilutions were used the figures represent the average of both. Since it required an entire day to perform a single experiment, for frequently as many as 120 tubes had to be seeded from a single animal, the same suspension of tubercle bacilli could not be used to inoculate all the rabbits of a given series. A given suspension was used to inoculate a number of rabbits corresponding to the number of intervals required, and similarly other suspensions, until the number of rabbits required for each interval was obtained. The slight variation in dosage thus introduced was lessened by preparing the suspensions as uniformly as possible from similarly aged cultures, etc. The same alkaline medium was used for making the suspension of tubercle bacilli as in suspending the tissues.

The Fate of Human Tubercle Bacilli in Large Dosage in the Various Organs of the Rabbit.

The number of colonies recovered from similar weights of lung, liver, spleen, kidney and bone marrow, 2 days, 1, 2 and 4 weeks and 2 months after injection of 0.1 mg. P-15 B into rabbits, after direct seeding upon Dorset's and Petroff's media and also after sodium hydroxide treatment following 3 months incubation, is recorded in detail in Table I.

TABLE I.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.1 Mg. B. tuberculosis, Human.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
9-75	2 days	—	—	—	—	—	23	0	0	78	—	10	15	—	0	—	0	5	2	—	—
9-76	2 days	—	?	17	—	42	22	—	6	200	300	—	34	—	2	0	0	6	11	0	3
9-82	2 days	—	?	—	—	28	60	1	0	23	25	0	0	0	0	0	0	26	2	0	1
Average	2 days	—	?	17	—	35	35	1	2	100	162	5	16	0	1	0	0	12	5	0	2
9-77	1 wk.	—	—	32	35	225	—	9	0	—	—	13	4	2	4	0	—	94	0	1	0
9-84	1 wk.	—	—	—	—	225	80	—	—	825	585	—	—	—	—	—	—	13	3	—	—
9-88	1 wk.	—	—	125	22	400	150	10	4	∞	875	62	37	31	5	0	0	64	58	1	1
Average	1 wk.	—	—	78	28	283	115	9	2	1,000	730	37	20	16	4	0	0	57	20	1	2
9-85	2 wks.	∞	∞	275	237	600	—	32	31	∞	∞	416	600	87	172	0	0	100	96	1	0
9-90	2 wks.	∞	∞	18	20	100	—	1	1	∞	∞	2	1	—	—	0	—	—	—	7	4
9-78	2 wks.	∞	∞	275	50	—	—	22	13	∞	1,185	5	0	—	37	9	0	333	75	33	4
Average	2 wks.	∞	∞	189	102	350	—	18	15	∞	∞	141	200	87	104	3	0	216	85	14	3
9-79	4 wks.	∞	∞	420	250	640	11	2	2	700	258	1	16	85	37	13	22	277	88	15	13
9-86	4 wks.	∞	∞	1,183	1,500	27	0	1	1	366	—	1	7	630	500	0	16	33	18	12	0
R 9-92	4 wks.	∞	1,200	—	—	—	—	0	0	300	20	—	1	545	283	—	4	57	16	1	4
Average	4 wks.	∞	∞	801	875	333	5	1	1	455	139	1	8	420	273	6	14	122	40	9	4
9-87‡	2 mos.	13,750	10,000	555	—	1	0	0	—	2	0	0	—	816	1,066	21	—	213	18	0	—
10-12‡	67 days	66	—	16	45	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
15-01‡	60 days	50	18	2	1	12	0.5	6	3	22	—	20	6	68	38	41	3	5	1	1	0
Average	2 mos.	4,622	5,009	191	23	4	0	2	1.5	8	0	6	3	294	368	20	1.5	72	6	0.3	0

* D. = Dorset's medium.
† P. = Petroff's medium.
‡ Computed from dilutions 1:10 and 1:100 instead of 1:6 as in all remaining figures in this table.

Mathematical accuracy is not to be expected in a biological experiment of this nature, especially in dealing with the capricious growth proclivities of an organism like the tubercle bacillus. Furthermore, variations in the susceptibility of individual rabbits, in the pieces of tissue selected, and in the dispersion of the tubercle bacilli in the inoculum, caused variations in the results obtained. The method is nevertheless adequate for the problem on hand.

The arithmetical averages of the number of bacilli recovered from each organ of three separate rabbits are plotted in the accompanying curves (Charts I and III).

Original Deposition.—2 days after the intravenous injection of bacilli, the number of colonies recovered from like quantities of the various organs of the rabbit is similar to the relative distribution of India ink after intravenous injection (8).
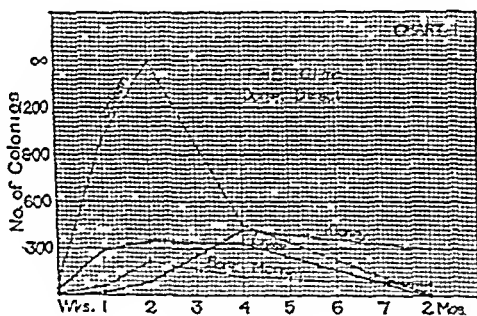
CHART I.

Thus from the spleen, an average of 100 colonies was recovered from 25 mg. of tissue on Dorset's medium, then 35 colonies from the liver, next 12 from the bone marrow, and none from the kidney. Essentially parallel results were obtained from Petroff's medium after direct seeding and from both media after sodium hydroxide treatment. Of course smaller numbers of colonies were recovered after treatment, but since we are not concerned with absolute but with relative quantities each of these data serves as a check for the other.

It is difficult to isolate tubercle bacilli in pure culture from the lung without sodium hydroxide, until pathological changes have taken place. For this reason it is not certain from the data in the table what is the relation of the lungs to the other organs as regards the deposition of tubercle bacilli. Apparently it is other than can be accounted for by the distribution of particulate matter.

1 week after the infection there is a rapid multiplication of the bacilli in the different organs, but not to the same degree in all the organs. The spleen shows

the most rapid multiplication; the liver much less.   An increased number of bacilli is also discernible in the bone marrow and kidney.

This increase in all the organs continues unabated in the *2nd week* after injection.
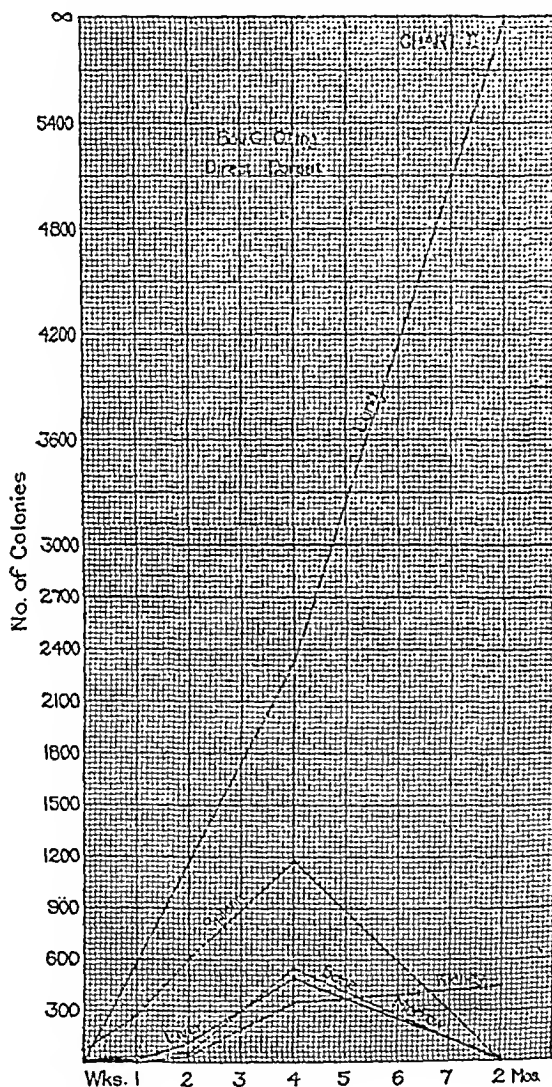
CHART II.

At this time, the relative numbers of colonies to be recovered from a given organ bear only a superficial relationship to the original deposition in that organ (see Table I).

4 weeks after infection, a striking change takes place. The picture differs greatly in the spleen, liver and bone marrow on the one hand, and in the lung and kidney on the other. In the first group of organs, especially in the spleen, there is not only a cessation of growth but a marked destruction of tubercle bacilli, as is indicated by the far fewer numbers of colonies recoverable from them after 4 weeks than after 2 weeks. There are still, however, large numbers of tubercle bacilli remaining in these organs. In the lung and kidney, on the contrary, the increase in the number of bacilli continues unabated.

2 months after infection the destruction is almost complete in the liver and spleen and comparatively few bacilli can be isolated from the bone marrow. But it is apparent that the rate of destruction between 4 and 8 weeks is much slower than
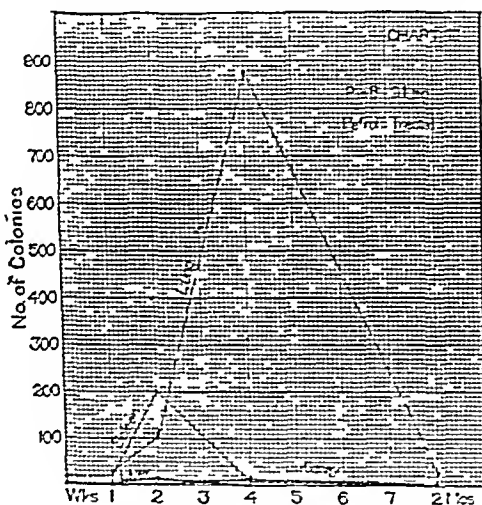
CHART III.

between 2 and 4 weeks. Destruction also becomes apparent in the lung, and cessation of growth, if not destruction, in the kidney.

The factor of individual resistance of the rabbit to tuberculous infection is especially noticeable in the 2 month interval. Compare the number of colonies isolated from the several organs of Rabbit 98-7 on the one hand and Rabbits 10-12 and 15-01 on the other. Such variations sometimes make the interpretation of data difficult.

It is seen then, that during the first 2 weeks after infection with 0.1 mg. of P-15 B the tubercle bacilli multiply in all the organs. Although their rate of increase differs in the different organs, there is as

TABLE II.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.1 Mg. B. tuberculosis, Bovine.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
9-98	2 days	—	—	0	0	10	15	0	0	66	7	—	0	0	0	0	0	0	0	0	0
17-62‡	2 days	5	0	0	0.8	10	0	4	4	70	0	0	2	—	—	0.5	0	20	0	0	0.5
Average	2 days	5	0	0	0.4	10	7	2	2	68	3.5	0	1.0	0	0	0.2	0	10	0	0.2	0.2
9-94	1 wk.	—	—	—	0	—	0	0	0	57	4	0	1	0	0	0	0	18	2	1	0
9-99	1 wk.	—	—	—	0	20	0	1	0	261	0	0	0	1	1	0	0	78	0	1	0
10-00	6 days	—	—	0	0	3	1	0	0	501	5	0	0	1	1	0	0	1	0	0	0
Average	1 wk.	—	—	0	0	12	1	1	0	273	3	0	1	1	1	0	0	32	1	1	0
9-95	2 wks.	—	—	2	2	118	0	1	3	416	1	0	0	—	—	0	0	49	1	0	1
10-01	2 wks.	—	—	12	45	61	0	1	10	800	0	1	3	22	44	3	0	28	17	0	1
10-05	2 wks.	—	—		0	180	0	1	0	600	0	0	0	57	0	0	0	88	0	1	0
Average	2 wks.	—	—	5	16	119	0	1	4	605	1	1	1	39	22	1	0	55	6	1	1
99-6	4 wks.	795	0	46	0	27	0	23	12	225	0	3	0	4	0	0	0	167	1	—	0
10-02‡	4 wks.	1,200	1,200	16	105	34	2	0	33	918	100	3	4	241	0	0	0	103	100	1	7
10-06‡	4 wks.	5,000	12,200	330	383	1,396	216	65	33	2,375	0	33	166	798	0	0	0	1,385	276	40	120
Average	4 wks.	2,331	4,466	130	162	485	72	29	15	1,172	33	13	57	347	0	0	0	551	125	20	42
9-97‡	2 mos.	1,666	1,120	233	85	27	0	4	3	16	0	0	0	21	0	4	1	0	0	0	0
11-59‡	64 days	∞	∞	∞	75,000	6	0	0	0.5	2	0	3	0	883	510	595	945	0.5	0	0	0
Average	2 mos.	∞	∞	∞	∞	16	0	2	1.7	9	0	1.5	0	452	255	299	473	0.2	0	0	0

* D. = Dorset's medium.
† P. = Petroff's medium.
‡ Computed from figures for 1:10 and 1:100 dilutions; all other figures represent dilution 1:6.

yet no effective opposition to the growth of the virus. Furthermore, the subsequent arrest in multiplication followed by destruction of the bacilli takes place about simultaneously in the three physiologically and anatomically very different organs, the spleen, liver and bone marrow. The only function these organs have in common is to remove particulate matter or bacteria from the blood stream, a func-
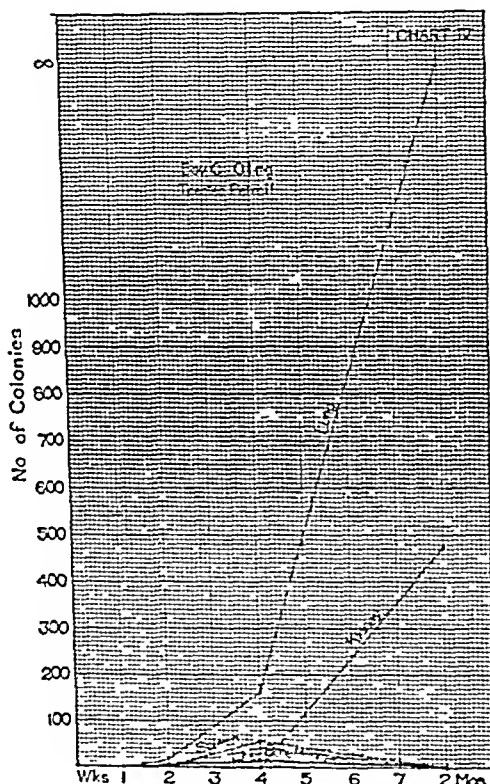
CHART IV.

tion shared to a much smaller degree by the lung and kidney, where destruction takes place later. Again this turn in the fate of tubercle bacilli does not depend upon the quantity of tubercle bacilli accumulated in a given organ. Thus in the spleen they reach uncountable numbers before destruction begins but only moderate numbers in the liver and bone marrow.

The majority of the animals recovered from the disease at the end of 2 months, though they were far from completely eliminating the bacilli.

The Fate of Bovine Bacilli in Large Dosage in the Various Organs of the Rabbit.

Table II and Charts II and IV show the fate of bovine tubercle bacilli in various organs of the rabbit at different intervals of time after the intravenous injection of 0.1 mg. of Bovine C, which is comparable in all respects to the fate of P-15 B.

One striking difference is apparent at once: *2 days* after bovine infection far fewer colonies were isolated from like quantities of tissue. Yet the relative distribution in the various organs is again found to be similar to that of particulate matter; namely, spleen, liver, bone marrow and kidney. In Rabbit 17–62 the deposition in the lung was less than in the bone marrow.

It was found that the growth *in vitro* of this virulent bovine organism is much more subject to unfavorable influences than the human type P-15 B. Yet when these two strains are transplanted on glycerol agar in the usual way, they grow equally fast and luxuriantly. It is possible that this difference in behavior may be attributed not to different *in vivo* relations but simply to the original dysgonicity of the bovine bacillus, which comes into play when the bacilli are sufficiently dispersed. It may be for this reason that fewer colonies are isolated from tissues infected with Bovine C, and not because fewer viable bacilli are present. This problem is being investigated separately. However, as can be seen from the tables, very large numbers of colonies can be isolated even from Bovine C at times.

1 week after infection there is an increase in the number of colonies to be recovered from the various organs, but the relative rise is much less than with P-15B.

Without attaching too much significance to the actual numbers, it is permissible to draw attention here to the following relationships. 1 week after infection, 5 days after the first interval, the human strain, seeded directly on Dorset's medium, showed an increase of about eight times in the liver, ten times in the spleen, five times in the bone marrow, and sixteen times in the kidney, while the bovine strain similarly cultured showed hardly any increase in the liver and kidney, and an increase of three times in the bone marrow and four times in the spleen.

Now even if we admit that the bovine strain is dysgonic on reisolation from the tissues so that we start with a lower base line for this strain, still if its rate of growth in the body were the same as the human strain's, the relative increase would be the same in the various organs. Therefore we are forced to the conclusion that during the 1st week the bovine strain grows more slowly in the body of the rabbit than the human strain.   With both strains the rate of multiplication of the bacilli in the spleen is much faster than in the liver.

2 weeks after infection the increase continues in all the organs, just as with the human strain.

The bovine bacilli reach their highest numbers in the liver, spleen and bone marrow *4 weeks* after infection, at a time when the human bacilli show a decided decrease.

A large percentage of the rabbits thus infected died before the close of the next interval.   Out of seven rabbits, only two survived 2 months.   The remaining five died on an average of 38 days after infection.   Two died on the 24th and 25th day after infection, at a time when the multiplication of the bacilli in all of the organs was going on unchecked.   Thus it is seen that, with large doses of bovine tubercle bacilli, death may take place before any effective opposition to the growth of the parasite in any of the organs is realized.   Individual rabbits of greater resistance may survive long enough for the altered reaction of the tissues toward the virus to become apparent.

2 months after infection we find the disappearance, almost but not quite complete, of the tubercle bacilli in the liver, spleen and bone marrow, the organs that showed the earliest change in behavior toward the human infection.   At the same time the animal is dying from pulmonary and renal infection.   For in contradistinction to the fate of the human strain, the lung and kidney still show an unhindered multiplication, especially the lung, where the tissue becomes veritably a pure culture of tubercle bacilli.   Thus even the resistant animal succumbs.

The chief difference between the human and bovine types, therefore, is the slower multiplication of the bovine bacilli and the later appearance of their destruction.   Although the difference is quantitative rather than qualitative it is sufficient to cause death after bovine infection, because of the continued multiplication in the lung and kidney, and regression of the lesion and survival after human infection.

The Fate of Human Bacilli in Small Dosage in the Various Organs of the Rabbit.

The human tubercle bacillus, then, grows more rapidly in the body of the rabbit than the bovine, and this more rapid growth is followed

TABLE III.

Number of Colonies Obtained from Organs of Rabbits Injected with 0.001 Mg. B. tuberculosis, Human, per Kilo.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.t	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
11-40	30 min.	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
11-61	30 min.	30	0	5	5	3	1	1	0	6	3	1	1	0	0	0.15	0	0	0	0	0
11-71	3 hrs.	3	2	0	1	0	0	0	1	1	0	3	2	0	0	0	0	0	0	0	0
11-62	1 day	—	—	1	1	2	0	1	2	4	1	1	1	0	0	0	0	0	0	0	0
12-25	1 day	9	11	5	2	1	0	2	3	9	10	7	10	0.15	0	0	0	0.15	0.3	0.3	0.15
11-46	2 days	—	3	0	0.5	1	0	0	0	3	3	0	3	0	0	0	0	0	0	0	0
12-28	2 days	15	9	3	6	—	0	2	5	8	3	3	3	0	0.15	0	0	0.15	0	0	0
Average	Short intervals	11.4	4.1	2.0	2.2	1.5	0.1	0.8	1.5	4.5	2.4	2.1	2.4	0.0	0.0	.	0.0	0.0	0.0	0.0	0.0
11-42	1 wk.	3	3	0	0	—	0	0.3	0	8	0	0	0	0	0	0	0	0	0	0	0
11-47	1 wk.	6	6	3	0	3	0	0.3	0.3	6	6	0.6	1.6	0	0	0	0	0	0	0	0
12-26	1 wk.	43	105	9.5	3	3	0.3	0	0	29	10	4.3	2.6	0	0	0	0	8	1.3	0.3	0.6
Average	1 wk.	17	38	4.1	1	3	0.1	0.2	0.1	14.3	5.3	1.6	1.4	0	0	0	0	3.3	0.4	0.1	0.2
11-48	2 wks.	63	50	33	15	12	0	2.6	1.3	211	30	11	18	0	0	0	0	0	0.3	0	0
11-74	2 wks.	—	—	46	47	30	34	14.8	8.3	844	390	150	143	0	1.8	0	1	17	16	1.3	4.5
10-86	2 wks.	870	330	78	129	88	86	9	16	1,850	1,630	244	180	1.3	0	0.3	0.3	25	32	14	11
Average	2 wks.	466	190	52	63	43	40	8.8	8.5	968	683	135	113	0.4	0.6	0.1	0.4	14	16.1	5.1	5.1
11-73	4 wks.	10,660	8,000	5,400	4,530	139	120	22	36	6,330	4,160	870	1,236	1.6	0	0.3	1.0	220	270	43	38
11-75	4 wks.	415	416	236	202	1.7	0	1	2	498	280	158	142	0	0	0	0	13	11	8	13
11-51	4 wks.	—	—	2,560	3,100	3	0	2	0	204	163	44	16	5	6.6	9	2	9	5.5	2.5	1.6
Average	4 wks.	5,537	4,208	2,732	2,610	47	40	8	12	2,344	1,534	357	464	2	2	3	1	80	95	17	17

No.	Age	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20
11-57	2 mos.	151	91	98	121	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
9-11	2 mos.	4,600	6,400	3,250	2,700	—	0	0	0	15	0	0	1.6	210	50	81	145	0	0	0	0
12-27	2 mos.	2,230	1,750	2,500	4,430	0	0	0	0.3	190	1.5	21	35	246	196	180	280	0	0	0	0
Average	2 mos.	2,327	3,748	1,919	2,417	0	0	0	0.1	69	0.5	7	12	152	82	87	141	0	0	0	0
13-09	4 mos.	—	—	—	0.5	–	–	0	0	–	–	0	0	–	0	0	0	0	0	0	0
13-19	4 mos.	—	785	690	390	–	–	–	0	0	0	—	1	–	1	0	0	0	0	0	0
11-18	4 mos.	—	20	17	0	0	–	0	0	1	1	0.3	2	–	0	0	0	1	1	0	0
Average	4 mos.	—	402	353	130	0	–	0	0	0	0	0.15	1	–	0	0	0	0	0	0	0
14-56	6 mos.	—	200	28	21	0	0	0	0	52	33	33	9	0	0	0	0	0	0	0	0
14-52	6 mos.	50	53	1	29	0	0	0	0	36	2	37	45	1	0	0	0	0	0	0	0
11-17	6 mos.	10	—	3	—	0	1	0	1	0	—	0	—	0	1	0	1	0	1	0	1
Average	6 mos.	30	126	15	25	0	0	0	0	29	17	23	27	0	0	0	0	0	0	0	0

* D. = Dorset's medium.
† P. = Petroff's medium.

by a more rapid destruction. The question arose whether the phase of destruction would be deferred if the accumulation of human tubercle bacilli were retarded by injecting smaller doses.

A series of rabbits was inoculated intravenously with 0.001 mg. per kilo of a human strain, P-48 A. This dose was chosen because it was thought that human
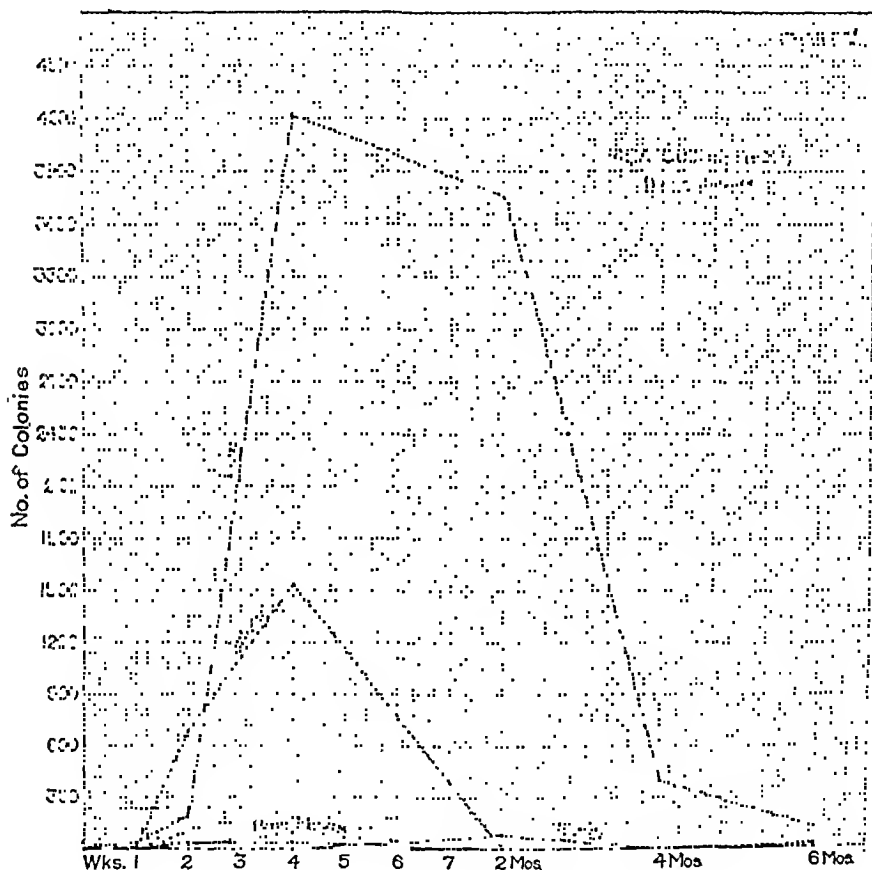
CHART V.

bacilli in a smaller quantity might be destroyed by virtue of the native reaction, and this strain, to see whether the relations would hold for a different strain.

The animals were killed at the following intervals: 30 minutes, 3 hours, 1 and 2 days, 1, 2 and 4 weeks, 2, 4 and 6 months. With the exception of the short periods at least three rabbits were used for each interval. The organs were cultured as above described. The detailed results are given in Table III and the averages are plotted in the curves of Chart V.

Original Distribution.—During the first *2 days* the greatest number of colonies was recovered from the lung, next from the spleen, next from the liver, and as a rule none from the bone marrow and kidney. No significant difference in distribution was noted if the rabbits were killed 30 minutes, 3 hours, 1 day or 2 days after infection. It is noteworthy that again, as apparently with the large doses of human bacilli, more colonies are recovered from the lung than from the spleen and liver, a distribution consistently at variance with the distribution of particulate matter. The relative distribution to the other organs follows that of manganese dioxide and carbon, namely, spleen, liver, bone marrow and kidney. Since even 30 minutes after injection the bacilli were recovered in greater numbers from the lung than from the other organs, the phenomenon cannot be due to a rapid and immediate multiplication in the lung and a slower initial multiplication or more rapid destruction in the other organs, but must be due to a greater deposition in the lung. No explanation can well be offered with the data at hand.

1 week after infection there is scarcely any rise in the number of bacilli recoverable from the various organs. This is in contrast to the sharp rise in the 1st week after the injection of 0.1 mg. P-15 B, and shows the same latent period noted after the injection of 0.1 mg. Bovine C.

2 weeks after infection there is a marked rise in the number of bacilli recoverable from the spleen and lungs. In the liver and bone marrow the increase is much less, and it is scarcely perceptible in the kidney. Here the striking difference in the behavior of tubercle bacilli in the several organs comes to the fore. The slight rise in the bone marrow and in the kidney can easily be attributed to the very slight original localization of bacilli in these organs. But the increase in the liver cannot, for here the original deposition was only 3 to 4 times less than in the spleen, and by the end of the 2nd week an average of 968 colonies was recovered from the spleens of three animals on Dorset's medium as against an average of only 43 colonies from their livers.

4 weeks. At this interval not only is there no destruction of the tubercle bacilli, such as was found in the spleen, bone marrow and liver 4 weeks after the introduction of 0.1 mg. P-15 B, but the bacilli are increasing in all the organs. However, the rate of increase in the interval from the 2nd to the 4th week is much slower than in the interval from the 1st to the 2nd week. It is noteworthy that the peak is reached in the spleen with over 2000 bacilli, but in the liver with only 47, showing again that the liver of the rabbit is far more unfavorable soil for tubercle bacilli than the spleen. Tremendous numbers of tubercle bacilli are recovered from the lung.

2 months after infection there is complete destruction of the bacilli in the liver and bone marrow, a marked reduction in the number recoverable from the spleen, and a considerable reduction in the lung. The drop in these organs appears to be simultaneous but probably only because of the length of time between intervals. It was unfortunately not forseen that observations 6 weeks after infection would have been desirable. In the kidney, the bacilli are still on the increase. It is

noteworthy that with the much smaller quantity of human bacilli, 0.001 mg., the destruction is not so complete at 2 months in the spleen as with the much larger dose, 0.1 mg. With the latter, an average of only eight colonies was recovered from the spleens of three rabbits at this time, but with the small dose an average of 69 colonies was recovered from somewhat smaller quantities, 16 mg., of the spleen on Dorset's medium. On the other hand the destruction is more complete in the bone marrow than with the larger dose.

Thus the chief differences appear in the behavior of a small quantity of human tubercle bacilli as compared with a large quantity of an organism of the same type though not the same strain. (1) There is a latent period of a week with the small dose; with the large dose the increase is immediate. (2) The destruction of tubercle bacilli begins later with the small dose than with the large dose. (3) At a given time after infection, more complete destruction is apparent after the large dose than after the small dose, at least in the spleen. Some organs, such as the liver and bone marrow, will show only slight numbers of bacilli even when their numbers are at their height after the small dose; after the larger dose the same organs will show considerable numbers.

Since none of the animals infected with the small dose of bacilli died from their tuberculous affection it seemed advisable to follow the fate of the bacilli in the various organs to their extinction, if possible.

4 months after infection the bacilli had been greatly reduced in the lung; they could no longer be isolated from the liver, kidney and bone marrow. Only a single stray colony could be recovered occasionally from the spleen, in which again, even 4 months after infection, the destruction of bacilli is not quite complete. Unfortunately these organs were cultured during the summer heat and many tubes appeared to be incompletely sterilized, although the methods used had heretofore been totally satisfactory in securing sterile media. However, sufficient data were obtained to assure us of the general trend of events.

6 months after infection the number of bacilli recoverable from the lungs is still further reduced. It is noteworthy that in the interval between the 2nd and 4th months the rate of destruction of the tubercle bacilli in the lung is very much faster than during the 2 months that follow. Thus on Petroff's medium after direct seeding the decrease in the 2nd to the 4th month was about 9 times, during the next 2 months a little more than 4 times. From the liver, kidney and bone marrow no tubercle bacilli were isolated 6 months after infection, just as none were recovered from these organs 4 months after infection. However, in the spleen a second slight increase became apparent.

Thus whatever force is responsible for the change bringing about the destruction of the tubercle bacillus weakens as time progresses. As a result the destruction is rapid at first, and becomes slower with time, and as early as 6 months after infection with 0.001 mg. P-48 A per kilo the bacilli have again begun to increase in the spleen while they are still decreasing in the lung. Possibly an adaptation of the parasite to the host has been effected.

The Fate of Bovine Tubercle Bacilli in Small Dosage in the Various Organs of the Rabbit.

A series of animals was infected intravenously with 0.001 mg. Bovine C per kilo. The detailed findings are presented in Table IV, and the averages are plotted in the accompanying curves, Chart VI.

With the smaller as with the larger dosages, fewer tubercle bacilli were isolated from the organs of rabbits infected with the bovine, than from those infected with the human strain. In harmony with this, and also with the relative distribution of particulate matter, the tubes seeded with suspensions of bone marrow and kidney 1 and 2 days after bovine infection with small doses, remained sterile in each case, and only occasionally and irregularly was a stray colony isolated from the lung and liver.

1 week after infection a slight rise in the number of colonies recovered was perceptible in the lung and spleen, while the liver, kidney and bone marrow gave practically all negative results in each of the three rabbits. This essentially parallels the latent period observed with a similar dose of a human strain.

2 weeks after infection there is a considerable rise in the number of colonies recoverable from the various organs. But the rise is much slower than with human bacilli in the same dosage. Thus between the 1st and 2nd weeks after infection with the human bacilli in small doses, the increase in the number of colonies recovered from the spleen was 69 times on Dorset's medium and 136 on Petrofi's medium after direct seeding, but with the bovine, it was 8 times on Dorset's medium and 31 times on Petrofi's. Like relations obtained in the other organs. It is noteworthy that very few bacilli were recovered from the liver of rabbits infected with small doses of either human or bovine strain; with the latter the number was negligible. On the other hand, the bacilli attain large numbers in the spleen of even bovine-infected animals and reach very large numbers in the spleens of rabbits infected with human bacilli. This discrepancy is altogether out of proportion to the relative original deposition in these two organs and seems to justify the conclusion that even in the "native" state the liver of the rabbit can check the growth of the bacilli much more completely than the spleen. At the end of the 2nd week some multiplication is also apparent in the lung and bone marrow. The kidney, however, still fails to show any bacilli.

TABLE IV.

Number of Colonies Obtained from Organs of Rabbits Infected with 0.001 Mg. B. tuberculosis, Bovine, per Kilo.

Rabbit No.	Interval after injection	Lung				Liver				Spleen				Kidney				Bone marrow			
		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated		Direct		Treated	
		D.*	P.†	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.	D.	P.
11-83	1 day	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13-15	1 day	—	—	0	0	0	0	0	0	1.6	0	0	0	0	0	0	0	0	0	0	0
11-86	1 day	—	—	0.3	0.3	0	0	0	0	0.5	0	0	0	0	0	—	0	0	0	0	0
11-85	2 days	—	—	0	0.3	0	0	0	0	0	0	0.3	0	—	—	—	0	0	0	0	0
13-35	2 days	—	—	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
12-30	2 days	1	0	0	0	0.3	0	0	0	7.5	0	0	0	0	0	0	0	0	0	0	0
Average	1 and 2 days	—	—	0	0.1	0	0	0	0	1.7	0	0.0	0	0	0	0	0	0	0	0	0
12-34	1 wk. (8 days)	—	—	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	0	0	0
13-29	1 wk.	—	0	0.3	0.3	0	0	0	0	4.3	0	2.3	0	0	—	0	0	1.6	0	0	0
12-32	1 wk.	47	0	7.6	20	0	0	0	0	20	0	2.3	0	—	0	0	—	0.5	0	0	—
Average	1 wk.	47	0	2.5	6.7	0	0	0	0	8.5	0	0.7	0	0	0	0	0	0	0	0	0
12-17	2 wks.	—	22	1	2	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
13-30	2 wks.	—	20	3	1.8	0	2	0	2.3	5.6	0	0	0	0	0	0	0	0	0	0	0
12-33	2 wks.	39	40	10	24	30	0	0	0	185	93	22	33	—	0	0	0	9.5	10	0	0
Average	2 wks.	39	27	4.6	9.2	10	0.6	0.6	0.7	64	31	7.3	11	0	0	0	0	3.1	3.3	0	0
11-89	4 wks.	60	85	8.5	2.5	0	0	0	0	30	16	35	0	0	0	0	0	0.5	0	0	0
13-36	4 wks.	30	—	13	29	0	0	0	0	705	625	40	107	44	0	9	6.5	21	6	0	7
12-36	4 wks.	257	750	52	91	3	0	3	3	750	916	305	385	105	0	8	0	5	0	2	3
Average	4 wks.	115	417	24	40	1	0	1	1	495	519	126	164	49	0	5.6	2.1	8.8	2	0.6	3.3

12-38	2 mos.	800	600	583	500	—	0 / 0	—	330	90 / 325	195‡	—	6	340	177	0	0	0	0		
12-31	2 mos.	—	290	23	28	—	0 / 0	1	0	0	0	0	0	0	0	0	0	0	0		
12-37	2 mos.	70	—	4	13	0	0 / 0	0	1.5	1 / —	0	—	90	3	3	1	1	1.50			
15-26	2 mos.	1,155 / 4,165		197	770	0.6 / 0.1	0 / 0	0	110	45 / 81	49	105	32	85	45	0	0	0.30			
Average	2 mos.	675 / 1,785		202	328							52									
15-04	4 mos.	—	650	220	117	0 / 0	0 / 0	0	1	0 / 0	0	0	0	0	0	0	0	0			
15-08	133 days‡	5,250 / 6,000		1,400	2,800	0 / 1	0 / 1	0	4	0 / 1	0.3 / 2,160	1,000‡	605	965							
Average	4 mos.	5,250 / 3,325		2,310	1,458	0 / 0.5	0.5 / 0	0	2.5	0 / 0.5	0.1 / 1,080	500	302	482							

* D. = Dorset's medium.
† P. = Petroff's medium.
‡ Probable figure.

4 weeks after infection, the bacilli have reached their highest numbers in the spleen and bone marrow. The growth in the lung continues unabated. For the first time since infection the bacilli appear in considerable numbers in the kidney. They were isolated from the liver of only one rabbit. In general the fate of the bacilli of the bovine and human strains is similar at this time, with, however, the following exceptions. With the human strain the number of bacilli in the lungs has gone far beyond those of the spleen. With the bovine strain the bacilli in
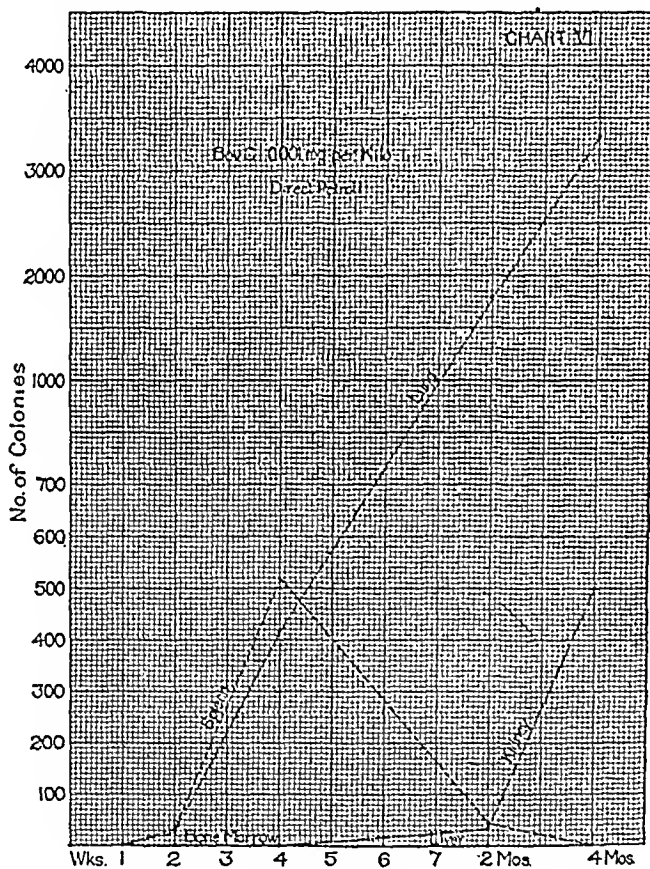
CHART VI.

the lungs still lag considerably behind those in the spleen, pointing again to the relatively slower multiplication of the bovine bacilli. Furthermore, the bacilli of the human strain reach higher numbers in the liver and bone marrow than do the bovine, so that the latter may be negligible while the former, even in the liver, attain considerable numbers. On the other hand, in the kidney the bovine bacilli have reached considerable numbers at this time, at least on Dorset's medium, whereas the human bacilli still are negligible.

2 months after infection the difference between the behavior of the human and bovine strains in this dosage is very striking.   In the human strain the lungs have not only checked the further growth of the bacilli but they show considerable reduction in their numbers.   The bovine strain, on the other hand, still continues to grow and multiply without any effective opposition.   In both cases the spleen has effectively and markedly reduced the number of viable bacilli recoverable from similar quantities of tissue; however, the destruction is much more complete in the spleen of the rabbit infected with the human strain than with the bovine strain.   With both types the bacilli have almost completely disappeared from the bone marrow and liver, in which the destruction is much more complete than in the spleen although these three organs generally parallel each other in behavior. In the kidney, both types of bacilli continue to increase.

4 months after infection the difference in the fate of the two types in small doses is most pronounced in two organs, the lung and kidney.   While the bacilli of the P-48 A strain continue to be destroyed in the lung in this period the bovine bacilli are still increasing in numbers.   Thus the drop in the lung begins in the 2nd month with the human infection and continues from then on, but the bovine bacilli continue to increase in number even 4 months after infection.   Again the kidney after human infection shows complete disappearance of the bacilli by the 4th month, while bovine bacilli continue to multiply in this organ unabated.   The destruction of bovine bacilli is also not quite so complete in the spleen as the destruction of human bacilli in the same interval.   However, in both instances, the destruction is quite complete in the liver and bone marrow.   The majority of the rabbits developed an extensive tuberculosis at the time of killing from which they would probably have died.

Thus we see, with the small doses of bovine as with the large doses, that one group of organs more or less completely destroys the bacilli— the liver, bone marrow and spleen—and the second group—the lung and kidney—is unable to check their growth.   As between the bovine and human strains in small doses, we see the same difference that appeared between the human and bovine strains in large doses: slower growth and slower destruction of the bovine bacilli.

DISCUSSION AND SUMMARY.

In this study the attempt has been made to follow the fate of tubercle bacilli in the lung, liver, spleen, kidney and bone marrow of rabbits infected intravenously with large and small doses of human and bovine tubercle bacilli by determining the number of colonies recoverable from similar quantities of tissue on egg media at varying intervals during the course of infection.   This method offers certain possibili-

ties for the elucidation of this problem precluded by the modes of attack used hitherto. Histological methods, while giving precise data in regard to tissue changes produced by the tubercle bacilli, are poor instruments for determining the fate of the bacilli in a given organ. Without stressing the notorious difficulties in staining the organism at all times, histological technique can give no definite answer to the question whether certain stained bacilli are living or dead, and it is the number of living bacilli that is of importance. Again animal inoculation, while an excellent index of the presence of living virulent bacilli, is a very inaccurate index of the number of living bacilli in a given specimen of tissue, for it is possible to infect guinea pigs with even a very few bacilli.

Lewis and Sanderson (9) have studied the histological changes in the rabbit's lung at varying intervals of time after the intravenous injection of similar quantities of human and bovine tubercle bacilli. They found that up to the 8th day tubercle bacilli are not abundant in either series. "After the 8th day bacilli become very difficult to find in the human series. In the bovine series they become increasingly numerous and very rapidly so." They conclude that "the natural resistance of the rabbit to infection with the tubercle bacillus of human type is apparently referable to a failure of this type of bacillus to multiply in the body of this species to any considerable extent. Exceptionally there are localized lesions (kidneys and lung nodules) which are associated with abundant growth and which show that this failure to multiply may be due to some positive growth restraining factor rather than to a failure of suitable nutritive materials." These authors, then, feel that the human tubercle bacillus does not grow very much in the body of the rabbit due to some undefined native restraining force.

Austin (10) approached this problem by treating rabbits inoculated intravenously with bovine and human bacilli with saline extracts of rabbit lungs. He found that fewer pulmonary lesions develop as a result of this treatment, especially in the bovine-infected group. He concludes that the "difference in the resistance of the rabbit lung to the two types of infection may be largely due to a quantitative difference in the defensive factors that are needed."

Rogers (11) studied the fate of human and avian bacilli in the liver of pigeons by histological methods after intravenous injection. He concluded that the bacilli are rapidly and extensively phagocytized by the endothelial cells lining the venous sinusoids of the liver and that the greater portion of them are rapidly destroyed by these cells in a time commensurate with the destruction of pneumococci. He found no evidence of mutiplication.

In summarizing the results obtained by cultural methods, it appears that the original localization of human or bovine tubercle bacilli in

the various organs of the rabbit follows the distribution of particulate matter (12), being greatest in the spleen, next in the liver, next in the bone marrow, and least in the kidney. The relative position of the lung among these organs is still doubtful owing to the difficulty of isolating tubercle bacilli in pure culture without treatment at the beginning of the infection. But in some work, to be described in a later communication, with 1 to 2 mg. of BCG given intravenously the deposition in the various organs of rabbits was found to be as follows: spleen 610, liver 322, lung 202, bone marrow 72 and kidney 6. These figures represent the number of colonies recovered from similar quantities of tissue after direct seeding on Dorset's medium 1 day after intravenous inoculation. They are in agreement with Bull's finding with typhoid bacilli (13).

The fate of the bacilli thus localized depends upon many factors: the dosage, whether large or small, the type, whether human or bovine, and doubtless many conditions not touched on here. Their original localization, although a factor, is far from being the controlling factor.

At first the tubercle bacilli grow and multiply in all the organs. The human type grows very fast, the bovine type much more slowly. Moreover, the rate of multiplication of both types is very different in the various organs. Thus in the spleen it is much more rapid than in the liver; it may even be negligible in the liver while very rapid in the spleen with small enough dosage, especially of bovine bacilli. But as yet in all the organs no effective opposition is offered to the multiplication of the tubercle bacillus.

Soon, however, a remarkable change takes place. The liver, spleen and bone marrow begin to destroy the tubercle bacilli. The time at which the destruction begins in these three organs depends on the dose and the type of bacillus. With large doses of human bacilli it becomes apparent between the 2nd and 4th weeks, when the bovine bacilli, in similar quantities, have reached their highest numbers. Their destruction does not become apparent until the interval between the 4th and the 8th weeks. If small quantities of human bacilli are injected, their destruction in these three organs is also delayed to the interval between the 4th and the 8th weeks.

By the 8th week after infection with a large dose of human bacilli the destruction is more complete than after a small dose. By the 8th

week after infection with a small quantity of bovine bacilli the destruction is much less complete in the spleen than after a small quantity of human bacilli. This change in the behavior of these three organs does not depend upon the actual number of bacilli accumulated in a given organ. All these facts speak clearly for the contention that the native power of the various organs to destroy tubercle bacilli is insufficient to check their growth effectually and, as a result of a stimulation most probably associated with the growth of the bacilli, an altered behavior toward the tubercle bacilli is brought about so that they are destroyed. However, while this change is sufficient in the liver and bone marrow to destroy the bacilli completely, it is insufficient to eliminate them completely from the spleen. This especially applies to small dosage.

Furthermore, the behavior of the tubercle bacilli in the lung and kidney is quite different from their behavior in the organs just mentioned. Here with large doses of human bacilli the destruction becomes apparent in the interval between the 4th and the 8th weeks instead of the interval of the 2nd to the 4th weeks as in the liver, spleen and bone marrow. With bovine bacilli in similar dosage the bacilli in the lung and kidney continue to increase in number until the death of the animal. With small doses of human bacilli the lung shows a beginning of destruction by the 8th week, when there is almost complete destruction in the liver, spleen and bone marrow. By the 4th month they have completely disappeared from the kidney and they are still decreasing in the lung. With the same dosage of bovine bacilli the organisms continue to multiply in the lungs and kidney even 4 months after infection. It is noteworthy that throughout the study it was found that after the phase of destruction has been brought about, the rate of destruction in the several organs is at first very rapid, but soon the force spends itself so that as time progresses the bacilli are more and more slowly destroyed, and a few lingering bacilli remain alive. In fact, in the spleen, 6 months after infection, a slight secondary rise was noticed. It is to be emphasized that the group of organs where tubercle bacilli are first destroyed is the liver, spleen and bone marrow, whose common function is to remove foreign particulate matter or bacteria from the body; and again, that of these three, the spleen lags behind the bone marrow and liver in its destruction of both bovine and human bacilli.

In the study cited above (1) on the cellular reaction of the organs of the rabbit toward tubercle bacilli it was found that the normal, non-immune rabbit destroyed heat-killed tubercle bacilli most effectively in the liver, less effectively in the bone marrow, much less in the spleen, and least of all in the lungs.

A correlation is thus seen between the native reaction of the several organs toward dead bacilli and the rate of destruction of living human and bovine bacilli in the same organs. The liver and bone marrow, which destroyed bacilli more effectively than the spleen in the "native" state, also destroy them more completely in the "immune" state, and these organs much more rapidly than the lung and kidney. Further-more, the native ability of the liver and bone marrow to destroy tubercle bacilli is almost sufficient to inhibit their growth altogether in rabbits infected with small doses of both the human and bovine types, especially the liver after infection with the bovine strain. Again throughout this study it was seen that tubercle bacilli, whether human or bovine, whether in large or small doses, always grow faster in the spleen than in the liver. This is perhaps to be connected with the liver's having a greater native ability than the spleen to destroy tubercle bacilli.

These facts offer some confirmation for the hypothesis (14) that "acquired resistance is only a specific increment of natural resist-ance." The natural resistance in itself is always insufficient to check the growth of the bacilli in all the organs.

The acquired ability of the organs to destroy tubercle bacilli be-comes increased as a result of the growth of the bacilli and the accumu-lations of their products in the body. The acquired ability is then added to the natural ability. In this light we can understand, to some extent at least, the fate of the bacilli in the various organs. The work of Lewis and his collaborators (15, 16) on the different hereditary powers of resistance of guinea pigs to tuberculous infection and the correlation that they have drawn between antibody production and this resistance would point in the same direction.

Römer (17) has shown that the allergic state in tuberculosis as repre-sented by the tuberculin reaction can be brought about more quickly with larger doses of tubercle bacilli than with smaller doses. This finding has recently been confirmed by Lewis and Aronson (18).

Since it was found that the human tubercle bacilli grow faster than the bovine and that their destruction in the various organs is brought about earlier, furthermore, since with smaller doses of human bacilli the altered body reaction is brought about later than with larger doses, again, since the destruction is more complete after the same time in certain organs with large doses of both types of bacilli than with small doses, the conclusion is forced upon us that perhaps the virulence of a given type of tubercle bacillus for the rabbit is closely related to the original growth of the bacillus. This is an inverse relation. The more rapid the growth, the less virulent. For the more rapidly a given organism grows in the body the more rapidly is a resistance stimulated and the more rapidly are the bacilli destroyed. On the other hand, the more slowly an organism grows the later is this brought about. Thus in the bovine infection, while the bacilli are effectively destroyed in the liver, spleen and bone marrow, the unabated growth of bacilli in the lungs and kidney brings about the death of the animal by the replacement of tissue consequent upon the unchecked tubercle formation. Doan and Sabin (19) have recently pointed out that regression of the tuberculous process takes place in the bone marrow and, to a lesser degree, in the spleen while the disease progresses in the lung and kidney even in bovine-infected rabbits.

However, this immunity is not complete; even as late as 6 months after the infection the bacilli have not entirely disappeared from the lungs of rabbits infected with 0.001 mg. of human bacilli per kilo, and apparently they are still persisting in the spleen if not increasing.

The results obtained in the study, not yet published, of the fate of BCG in the various organs of the rabbit, are altogether in harmony with the conception that the virulence of mammalian tubercle bacilli for the rabbit is inversely related to their multiplication in the body. With these bacilli the process of quick growth followed quickly by destruction is even more rapid than with the human type.

It is pertinent to note that animals that are susceptible to the mammalian bacillus are usually more susceptible to the bovine type than to the human type as determined by experimental inoculation. Thus (20) the ox, sheep, goat, cat, pig and rabbit are much more susceptible to the bovine infection than to the human. Even the guinea pig and monkey are somewhat more susceptible to the bovine type than to

the human. The dog is apparently moderately susceptible to both, although some maintain that the dog also is more susceptible to the bovine type (21). The only species for which, it is thought, the human type is more virulent is man. But even this conclusion is doubtful (Neufeld (22)). For man is infected by the bovine bacillus largely by the intestinal route, for which much larger dosages are necessary than for the respiratory route, by which tuberculosis is far more commonly spread among human beings. It is possible, therefore, that the mechanism of virulence may be the same in all species.

CONCLUSIONS.

1. The original distribution of tubercle bacilli of both human and bovine types to the various organs of the rabbit after intravenous inoculation follows the distribution of particulate matter in the following order per gm. of tissue; spleen, liver, lung, bone marrow and kidney. The relative position of the lung amongst these organs is less certain than that of the others.

2. At first the tubercle bacilli both of the human and bovine types grow in all the organs without any effective opposition.

3. The rate of growth of both types differs in the various organs. It is much faster in the spleen than in the liver. With small doses very little growth takes place in the liver and bone marrow, especially with the bovine type.

4. The human type of tubercle bacillus grows faster in the several organs of the rabbit than the bovine type.

5. This more rapid growth of the human type is followed by an earlier and more complete destruction of the human type than of the bovine.

6. With both types destruction occurs first in the liver, spleen and bone marrow. In the lung and kidney destruction of the human type takes place later, and unchecked multiplication of the bovine type continues in these organs until the death of the animal.

7. With smaller doses of human bacilli destruction is brought about later in the liver, spleen and bone marrow than with larger doses. With both types, in a given time, destruction is more complete in some organs after a large dose than after a small dose.

8. The destruction in the various organs is rapid at first and pro-

gresses more slowly as time passes. So that even 6 months after intravenous injection of small doses of human tubercle bacilli they have not yet completely disappeared from the lung and spleen.

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AGGLUTINATION BY PRECIPITIN.

Second Paper.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

In a previous paper[1] attention was called to the fact that serum heated sufficiently to cause turbidity reacts more markedly on the addition of precipitin than unheated or slightly heated serum under the same conditions. The intensity of the reaction was explained on the basis that a portion of the proteins had been coagulated by heat and the coagulated particles had become covered with a film of undenatured proteins remaining in solution with the result that they agglutinated on the addition of antibody. Evidence confirming this view was provided by experiments in which bacteria or collodion particles were mixed with antigens and agglutinated when specific precipitin was added. It was further shown that collodion particles exposed to cow serum or crystallized egg albumin and subsequently washed until the wash fluid no longer contained the antigen agglutinated when the specific precipitin was added. Loeb[2] had previously found that collodion particles treated with proteins acquire a film of the protein on their surfaces and in consequence manifest the cataphoretic behavior of proteins. This being the case the particles employed in the writer's experiments behaved in a manner similar to suspensions of bacteria or other particulate antigens when mixed with specific antisera.

The character of the deposition on the particles is of considerable interest. From the experiments previously reported one gains the idea that the surfaces of particles exposed to protein become entirely covered with the substance. The present experiments show that this is not the case.

[1] Jones, F. S., *J. Exp. Med.*, 1927, xlvi, 303.
[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.

EXPERIMENTAL.

Since the procedure was essentially the same in all the experiments it may be said that for purposes of "sensitization" small quantities of heavy aqueous suspensions of collodion particles were mixed with antigens and incubated for various intervals. After incubation an excess of 0.9 per cent NaCl solution containing 2 cc. of N/20 NaOH per 100 cc. was added. The mixtures were then centrifuged at high speed, the supernatant discarded, and the sediment resuspended in alkaline salt solution. The process of centrifugation and washing was repeated twice and the particles were finally suspended in the alkaline salt solution. With this method relatively stable test fluids were obtained.

TABLE I.

The Effect of Antigenic Concentration on the Sensitivity of Collodion Particles as Judged by Agglutination.

Particles exposed to various concentrations of crystallized egg albumin	Dilutions of anti-egg albumin serum						Control[*]
	1:10	1:20	1:50	1:100	1:200	1:500	
per cent							
10	C[**]	C	C	++++	++	+−	−
5	C	C	C	+++	+	+−	−
2.5	C	C	C	+++	+	+−	−
1	C	C	C	++	+	−	−
0.5	C	C	++	++	+−	−	−

[*] Throughout the experiments the control tubes contain only suspensions of sensitized collodion particles.

[**] Agglutination has been indicated as follows: C = complete; ++++ marked agglutination without complete clearing of the fluid; +++ well defined deposition; ++ moderate sedimentation; + slight deposition; +− a trace of sediment.

The precipitins had been produced by the immunization of rabbits or fowls with various antigens. Some were freshly obtained, others had been on hand for several months.

Hitchcock[3] has shown that collodion membranes when in contact with solutions of crystallized egg albumin adsorb the protein on their surfaces. The amount of protein adsorbed is dependent within certain limits on the concentration of egg albumin in the solution. In the previous work reported by the writer the concentration of protein was not varied. It seemed advisable to determine first to

[3] Hitchcock, D. I., *J. Gen. Physiol.*, 1925–27, viii, 61.

what extent the concentration of protein used for the purpose of sensitization of the collodion particles would influence subsequent agglutination. Experiment 1 was devised to throw light on this point.

Experiment 1.—To 10 cc. of 10, 5, 2.5, 1, and 0.5 per cent solutions respectively of crystallized egg albumin the same quantity of a suspension of collodion particles was added. The tubes were incubated for $1\frac{1}{2}$ hours and the particles washed. After the final centrifugation the particles were suspended in alkaline salt solution and tested with anticrystallized egg albumin. The protocol in Table I is typical for such experiments. For control purposes sensitized particles were tested with normal rabbit serum, but since no agglutination occurred the results are not recorded in the table.

The antiserum agglutinated the particles in about the same titer in the first three series. This suggests that little more antigen is adsorbed by the particles from a 10 per cent solution of crystallized egg albumin than from one containing only 2.5 per cent. When the antigen concentration employed for sensitization fell below 2.5 per cent there was a slight decline in the agglutination titer although the exposure to even a 0.5 per cent solution of crystallized egg albumin resulted in well marked agglutination in the presence of specific antibody. The influence of concentration on adsorption corresponds roughly with Hitchcock's findings obtained by direct weighing of the protein taken up by collodion membranes.

On the basis of this experiment one might suppose that provided sufficient protein exists in the sensitizing fluid the particles will become completely covered and,—since an excess of protein cannot cover them more,—will react on the introduction of antiserum. Again it is possible that regardless of the concentration, provided there be sufficient protein available, the particles would adsorb only limited amounts of the antigen. Experiments dealing with this phase of the question follow.

Experiment 2.—Collodion particles were added to 5 cc. of cow serum and to a similar amount of 2.5 per cent crystallized egg albumin. The tubes were then incubated for 1 hour. After incubation an excess of alkaline salt solution was added to each tube and they were shaken and centrifuged at high speed. The supernatant was then withdrawn and the particles originally exposed to cow serum were resuspended in 5 cc. of 2.5 per cent crystallized egg albumin. The particles from the tube previously exposed to crystallized egg albumin were resuspended in

cow serum. After further incubation of 1 hour an excess of salt solution was added and the tubes were centrifuged. The particles were then washed twice by centrifuging in alkaline salt solution, suspended in alkaline salt solution, and tested for the presence of adsorbed protein by the addition of anti-cow and anti-egg albumin serum. The results are given in Table II.

Table II indicates that regardless of the order in which the particles were sensitized about the same quantity of protein was adsorbed since the particles agglutinate about as well with either precipitin. That the particles are not completely covered by the protein seems true since the reaction of the particle after sensitization to a second antigen

TABLE II.

The Effect of the Exposure of Collodion Particles to One Protein after Another.

Treatment	Tested with	Dilutions of serum						Control
		1:10	1:20	1:50	1:100	1:200	1:500	
Cow serum followed by crystallized egg albumin	Anti-cow serum	++	C	C	C	C	++	+−
	Anti-crystallized egg albumin	+++	+++	C	C	C	++	+−
	Normal rabbit serum	+	+−	+−	+−	+−	+−	+−
Crystallized egg albumin followed by cow serum	Anti-cow serum	++	C	C	C	++++	+	+−
	Anti-crystallized egg albumin	++	+++	C	C	++++	+	+−
	Normal rabbit serum	+−	+−	+−	+−	+−	+−	+−

is about the same as when this antigen was used primarily. Other experiments given later confirm these findings, indicating that the particles are not completely covered by the antigens.

It might be said that through the combination of particle and protein the latter was so changed that it would react with any anti-serum. Since all the experiments are dependent on the specificity of the agglutination, an experiment which covers this phase of the question is included.

Experiment 3.—Collodion particles were sensitized with 2.5 per cent crystallized egg albumin or cow serum by the usual procedure. Both lots were then washed

in alkaline salt solution until the last wash fluid no longer contained antigen. The particles after resuspension were tested with the antiserum specific for the sensitizing substance and with other precipitins in addition. Thus particles sensitized with egg albumin were tested with anti-egg albumin, anti-cow or anti-horse serum, and those sensitized with cow serum were tested with anti-horse and anti-cow serum. The protocol is given in Table III.

The reaction must be regarded as specific since the sensitized particles react only with the precipitin specific for the sensitizing substance.

It being true that the particles are not completely covered by the antigen and that the agglutination in the presence of antibody is

TABLE III.

The Effect of Homologous and Heterologous Precipitin on Sensitized Collodion Particles.

| Sensitized with | Tested with | Dilutions of serum | | | | | | Control |
		1:10	1:20	1:50	1:100	1:200	1:500	
Crystallized egg albumin	Anti-egg albumin	+++	++++	C	++++	++	+	−
	Anti-cow serum	−	−	−	~	−	~	−
	Anti-horse serum	−	−	−	−	−	−	−
	Normal rabbit serum	−	−	−	~	−	~	−
Cow serum	Anti-cow serum	C	C	C	++	+	+	−
	Anti-horse serum	+	+−	−	~	−	−	~

specific, it is of further interest to ascertain the effect of sensitizing the particles to several types of proteins and measuring the amount of fixation by agglutination with sera specific for the sensitizing antigen. Experiment 4 is designed to cover this phase of the question.

Experiment 4.—There were on hand five antisera and after a number of preliminary experiments it was decided to sensitize particles to the five antigens and ascertain by agglutination with the antisera to what extent the antigens had been adsorbed on the particles. The following procedure was adopted. A heavy suspension of particles was added to 5 cc. of the protein solution and incubated ½ hour. 3 or 4 volumes of alkaline salt solution was then added and the tubes centrifuged at high speed. The supernatant was withdrawn and 5 cc. of the next antigen was added to the residue and after mixing thoroughly incubated ½ hour.

This was repeated until the particles had been exposed to all five antigens. The particles were then washed and resuspended in salt solution and tested with antisera.   In the protocols given in Table IV the tests were made in the order in which the particles were sensitized.   For control purposes the sensitized particles were tested with normal rabbit and normal chicken serum, since immune serum from each species was employed in the experiment, but no agglutination occurred and the record is therefore omitted from the table.

It can be said that the particles adsorbed some of each protein. When the exposure was first to egg albumin and subsequently to the

TABLE IV.

The Agglutination of Collodion Particles Sensitized with Five Antigens.

Particles sensitized successively with	Tested successively with	Dilutions of antisera						Control
		1:10	1:20	1:50	1:100	1:200	1:500	
Crystallized egg albumin, rabbit serum, chicken serum, horse serum, and cow serum	Anti-egg albumin	++++	++++	++	++	+	+−	−
	Anti-rabbit serum	C	++++	++	+	−	−	−
	Anti-chicken "	++++	+++	+	+−	−	−	−
	Anti-horse "	++++	+++	+	+−	−	−	−
	Anti-cow "	++++	C	++	+	−	−	−
Cow serum, rabbit serum, horse serum, chicken serum, and crystallized egg albumin	Anti-cow serum	++++	+++	++	+−	−	−	−
	Anti-rabbit "	C	++++	+	−	−	−	−
	Anti-horse "	+++	+++	++	+	−	−	−
	Anti-chicken "	+++	++++	++	+	+	−	−
	Anti-egg albumin	++	+	+	+−	−	−	−

other antigens comparable agglutination occurred with all the antisera. If however the particles were mixed with the serum antigens first and finally treated with egg albumin, the agglutination titer indicated that little of the egg albumin was adsorbed.   Other experiments indicated that this was true.

When the antigens are mixed and suspended particles are added similar but not as decisive results are obtained.   This has been brought out in Experiment 5.

Experiment 5.—5 cc. of 2.5 per cent solution of crystallized egg albumin and the same amount of cow, chicken, horse, and rabbit serum were mixed and incubated

for 1½ hours with a heavy suspension of collodion particles. After incubation an excess of alkaline salt solution was added and the mixture centrifuged. The usual procedure was employed for washing and resuspending the particles. Portions of the suspension were then tested with the various antisera. The result is indicated in Table V.

It was to be expected that the fixation would be less marked when the antigens were mixed since they were diluted by each other, yet it was possible to show that some fixation actually took place between each substance and the particles. It was weakest with egg albumin and strongest with cow serum.

Since collodion particles are readily sensitized with proteins and agglutinated on the addition of specific precipitins it seemed possible

TABLE V.

The Effect of Sensitizing Collodion Particles with a Mixture of Antigens.

Antigen mixture	Tested with	Dilutions of antisera					Control
		1:10	1:20	1:50	1:100	1:200	
5 cc. each of 2.5 per cent crystallized egg albumin, cow serum, chicken serum, horse serum, and rabbit serum	Anti-cow serum	++++	C	+++	++	—	—
	Anti-egg albumin	++	++	+	—	—	—
	Anti-chicken serum	+	+++	+	—	—	—
	Anti-horse "	C	++++	++	+	—	—
	Anti-rabbit "	C	C	++	—	—	—

that they might adsorb from sera rich in antibody sufficient precipitin to agglutinate in the presence of specific antigen. A series of experiments designed to throw light on this question was undertaken. Experiment 6 is an example.

Experiment 6.—0.5 cc. of a suspension of collodion particles was added to 2 cc. of rabbit serum rich in precipitin for cow serum. The mixture was incubated 1¼ hours and then 5 cc. of alkaline salt solution was added and the whole centrifuged. The sediment was washed twice more in an excess of salt solution and finally suspended in 25 cc. of alkaline salt solution. The sensitized particles were then tested (1) with antigen (cow serum); (2) with anti-rabbit serum, to determine whether or not rabbit serum protein had been adsorbed by the particles; and (3) with normal rabbit serum, for control purposes.

In addition the first supernatant fluid containing 2 cc. of the anti-cow serum and

5 cc. of NaCl solution was tested with antigen to determine whether or not the antibody content of the serum had been diminished by the admixture of the particles. For control purposes the effect of the dilution of antiserum with salt solution is included.

TABLE VI.

The Effect of Antigen on Collodion Particles Sensitized with Serum Rich in Antibody.

	Amounts of cow serum added							
	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	None
Sensitized with anti-cow rabbit serum	+	+−	+−	+−	+−	+−	+−	+−

TABLE VII.

The Agglutination of Particles Sensitized with Immune (Rabbit) Serum by Anti-Rabbit Serum.

Tested with	Amount of antiserum added						Control
	1:10	1:20	1:50	1:100	1:200	1:500	
Anti-rabbit serum (fowl)	C	C	C	++	+	+	+−
Normal fowl "	+−	+−	+−	+−	+−	+−	+−

TABLE VIII.

The Effect of the Collodion Particles on the Precipitin Titer of the Anti-Cow Serum.

	Dilutions of antigen							
	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480
Serum incubated with collodion particles ·	++	++	+	+	+	+	+	+
Serum diluted to the same extent but no particles added	++	++	++	+++	++	++	++	+

The effect of the addition of antigen to suspensions of collodion particles first exposed to serum rich in antibody is shown in Table VI. That there was adsorption of rabbit serum protein on the particles is brought out in Table VII; also that the particles removed some of the antibody for the immune serum is shown in Table VIII.

The experiment reported is one of a series, in others of which attempts were made to sensitize particles to anti-egg albumin or anti-horse serum and to agglutinate the particles with the specific antigen, but in no instance were they successful. It could always be shown that some of the proteins of the immune serum combined with the particles since agglutination occurred when a serum specific for the proteins was added to the suspensions of the particles. It was also possible to show that the titer of the antiserum declined after incubation with the collodion particles.

DISCUSSION.

Mention has been made of Loeb's view that the reason why collodion particles mixed with proteins behave cataphoretically like the proteins is because of the deposition of a protein film on their surfaces. The experiments reported throw more light on this phenomenon. Further proof has been brought that there is deposited on the surface of the collodion particles a portion of the protein, for the particles even when washed agglutinate in the presence of a precipitin specific for the protein. The experiments in which particles were exposed to several proteins and subsequently agglutinated with all the respective antisera argue that the deposition is not in the form of a complete film. It is probable that the protein is deposited on the surfaces in adherent patches. If this were not the case the particles would react only with a single antiserum regardless of the number of antigens applied, since if it were possible to completely layer several proteins one above another the outermost only would come in contact with the antiserum.

It is generally conceded that agglutination is a surface phenomenon dependent on a quantitative union of antigen and antibody. Relatively little has been reported on how much antigenic surface is necessary for the appearance of the phenomenon. It can be said that since collodion particles sensitized to five proteins may react by agglutination about as well to all the specific antisera, it appears that if as little as one-fifth of the surface is antigenic agglutination will occur under the proper conditions.

The experiments in which the particles were first suspended in antisera with the idea that they might adsorb sufficient antibody on

their surfaces to agglutinate in the presence of antigen are of general interest. They adsorbed antibody from the serum and furthermore proteins of the serum containing the antibody could be detected on their surfaces, yet agglutination failed to occur when antigen was added. It may be true that insufficient antibody was adsorbed. Shibley's[4] conception of bacterial agglutination, based on his experiments and those of Northrop and De Kruif and De Kruif and Northrop,[5] affords an explanation of the failure of agglutination under these conditions. He contends that agglutination depends on a specific union of antibody with antigen with the result that the cell surface is coated with a film of globulin from the immune serum. This confers upon the organism the character of denatured globulin with a reduction of the charge by electrolytes to a critical level between 12 and 14 millivolts and clumping results. A similar explanation may be applied to the agglutination of sensitized particles in the presence of antiserum specific for the sensitizing protein. The experiments in which collodion particles exposed to antiserum and then washed failed to agglutinate when antigen was added seem to indicate that the steps are irreversible, since the experimental procedure precluded the deposition of the film of denatured globulin, the necessity of which Shibley recognizes.

SUMMARY.

The fact is shown that collodion particles sensitized with various proteins adsorb sufficient protein to agglutinate in the presence of precipitin specific for the adsorbed protein. The amount of adsorption, judged by agglutination, is not dependent on the concentration of the sensitizing protein beyond a certain maximum. The agglutination resulting from the addition of immune serum to particles sensitized with protein is immunologically specific.

Particles exposed to a number of antigenic substances in succession are agglutinated by all of the appropriate antisera.

Particles exposed to immune serum and subsequently washed fail to agglutinate in the presence of antigen although some of the protein constituents of the immune serum are fixed upon them and its antibody content diminishes.

[4] Shibley, G. H., *J. Exp. Med.*, 1926, xliv, 667.
[5] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655; 1922–23, v, 127, 139, 605.

THE EFFECT OF SOME OF THE CHEMICAL CONSTITUENTS OF TUBERCLE BACILLI ON THE PROTO-PLASM OF AMŒBA DUBIA.*

By PAUL REZNIKOFF, M.D.

(*From the Laboratory of Cellular Biology, Department of Anatomy, Cornell University Medical College, New York City.*)

PLATE 5.

The successful isolation of various chemical constituents from tubercle bacilli by Johnson and Coghill (1) and Anderson (2, 3) has afforded an opportunity of using these substances in biological experiments. Recently Sabin and Doan (4) have tested the chemical fractions on rabbits and found distinctly different reactions to these components on the part of the clasmatocytes and monocytes.

The action of the derivatives from the tubercle bacilli on *Amœba dubia* was studied with the micrurgical technique, the same procedure being used that has been described previously (5). Although there is no evidence that fresh water amebæ are associated with tubercle bacilli, the use of such material was considered desirable because the ameba represents a simple cell upon which many data have been gathered in this laboratory with respect to its reaction to various chemicals.

Immersion Experiments.

Protein.—Amebæ were immersed in suspensions of proteins 304, 304-A (from unautoclaved, defatted organisms), 304-B (from autoclaved, defatted organisms), 308, and Dr. Florence B. Seibert's culture medium protein.[1] These suspensions were brought with NaOH to a hydrogen ion concentration varying from pH 7 to pH 8.2, depending

upon their maximum solubility. As controls, suspensions of serum albumin were used. Amebæ, placed in such suspensions, and kept in the ice box, showed no change in motility, in ability to produce pseudopodia, or in longevity, compared to amebæ kept under normal cultural conditions for at least 5 days. This innocuous effect of the proteins is true even though some denaturation occurs. If the containers are permitted to remain at room temperature and the proteins become more quickly and completely denatured the amebæ lose their pseudopodia, their plasmalemmæ become stiff, and they become very sluggish.

Phosphatides.—Amebæ were immersed in emulsions of the phosphatides A-3 and A-4. The resulting hydrogen ion concentration varied between pH 5.5 and 6. Amebæ immersed in emulsions of A-3 died in 1 day in concentrations of 0.01 per cent, in 2 days in 0.003 per cent, and were not able to exist in a normal state for 5 days until a dilution of 0.0015 per cent was reached. In A-4 amebæ died in 3 days in a 0.006 per cent emulsion and were living and well for at least 5 days in a 0.003 per cent emulsion.

The manner of death of amebæ in the phosphatide emulsions is characteristic of that due to surface-dispersing action, such as is caused by soaps (6). A normal ameba (Fig. 1) is characterized by a distinct plasmalemma, varying degrees of pseudopodial formation, and, as judged by the rate of the movement of the granules, an actively flowing endoplasm and a more quiescent ectoplasm. When amebæ are placed in emulsions of phosphatide derived from tubercle bacilli, the plasmalemma becomes sluggish and unable to flow even though the cytoplasm is in active motion. The cell, therefore, forms no pseudopodia and becomes round. Gradually the plasmalemma loses its form, breaks in places, and disappears as though it were dissolved, leaving the naked protoplasm as solidified debris (Fig. 2).

With emulsions of lecithin and of lecithin and cholesterol as controls no evidence of toxicity was seen when amebæ were immersed in as strong a mixture as 0.06 per cent, which is the strongest emulsion compatible with visibility of the amebæ in such a thick suspension.

Fatty Acid.—The fatty acid derived from the phosphatide is immiscible with the aqueous medium containing amebæ. If, however, amebæ are brought to an adjacent drop of fatty acid with the micro

needle, the plasmalemma of the cell which is in contact with the fatty acid quickly disappears.

If the ameba is pushed back into the water the plasmalemma is reformed. In one case an ameba was snared rapidly out of the aqueous medium into the fatty acid droplet and the plasmalemma disappeared instantly. When this acid is dissolved in 95 per cent alcohol, it gives an indication of having a pH between 2.8 and 3.0.

Polysaccharide.—Amebæ immersed in a 1 per cent aqueous solution of the polysaccharide A-8 (pH 7 with NaOH) derived from tubercle bacilli show no toxic effects even after 4 days. The growth of fungi makes longer observations impossible.

Injection Experiments.

Protein.—Injections of moderately large amounts ($\frac{1}{3}$ of an ameba full) of suspensions of 304 and 308 render the amebæ sluggish for about 5 minutes. Smaller amounts ($\frac{1}{4}$ of an ameba full) have only slight effect and larger injections ($\frac{1}{3}$–$\frac{1}{2}$ of an ameba full) usually cause quiescence and death. Injections of serum albumin and of Dr. Seibert's culture medium protein were relatively without effect upon the amebæ. Fig. 3 shows an ameba injected with protein 304. All the granules stand out distinctly, indicating almost complete quiescence, as contrasted with the blur seen in the normal ameba represented by Fig. 1, indicating rapid movement during the time of photographic exposure.

Phosphatides.—Injections of phosphatide emulsions have no other effect than that seen if the solvent alone is injected.

Fatty Acid.—This substance is not readily miscible with protoplasm. When injected it forms a discrete sphere in the cytoplasm. Usually the ameba relegates the droplet to the rear and appears to be in the process of pinching it off with the surrounding protoplasm. Before this can be accomplished, however, the plasmalemma in the vicinity of the drop disappears. If a sufficiently large drop of fatty acid is injected, the plasmalemma of the entire ameba can be made to dissolve. If the nucleus approaches the drop, it becomes hyaline. In one case the nucleus flowed into the fatty acid droplet and became hyaline.

Polysaccharide.—Injection of as strong a solution of polysaccharide

A-8 as 1 per cent in large amounts had no more effect upon the ameba than injection of water.

<div align="center">DISCUSSION.</div>

The action of the chemical constituents derived from tubercle bacilli upon amebæ may give little indication of the exact physiological responses of other cells to these bodies. But the general effect of these bacterial substances on any particular part of the ameba gives a suggestion of the probable chemical reaction involved. It is interesting to note that the phosphatide and fatty acid act upon the surface of the cell and the protein affects the interior. The polysaccharide has no apparent action on the normal ameba.

One function of the apparently non-toxic polysaccharide, as far as its chemical significance is concerned, may be to make the fatty acid miscible with protoplasm when these two substances are combined as a phosphatide. What other functions, either chemical or immunological, the polysaccharide may have is beyond the scope of these experiments.

It is of interest to consider the possible relation this work may have to the action of the whole organism on the cell. If the phosphatide fraction is in the waxy outer coat of the organism and this is miscible with the plasma membrane of the cell, it is easy to conceive how the bacillus can penetrate into the cell. If the outer covering of the organism is digested once the bacterium is engulfed, the protein may be free to act on the internal protoplasm.

In these experiments there is no direct evidence that the substances used have some specific effect by virtue of their derivation from the tubercle bacillus. As a matter of fact, except for their slower rate of action, the phosphatide and the fatty acid act as all plasma membrane solvents such as soaps (6), CO_2, or lactates (7). There is some evidence that the proteins 304 and 308 are more toxic upon the internal cytoplasm than serum albumin or culture medium protein. But Dr. Seibert (8) finds that her protein gives a 5 + skin reaction as contrasted to a 3 + with 304. This question of specificity of action, therefore, needs further investigation. These experiments aim merely to indicate the general chemical reactions of the derivatives used upon a simple cell.

CONCLUSIONS.

1. Protein fractions derived from tubercle bacilli are toxic to the interior of *Amœba dubia* but have no action on the plasmalemma.

2. Phosphatide fractions dissolve the plasmalemma but have no effect on the internal cytoplasm.

3. The fatty acid fraction has a marked solvent action on the plasmalemma when brought in contact with the surface of the cell. When injected it may slowly penetrate the cytoplasm to dissolve the contiguous plasmalemma.

4. The polysaccharide fraction has no effect upon the surface membrane or upon the internal cytoplasm of *Amœba dubia*.

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6. Reznikoff, P., Micrurgical studies of soaps, glycerine, dextrose and ethylene glycol on *Amœba proteus*, *Proc. Soc. Exp. Biol. and Med.*, 1927, xxiv, 380.

7. Reznikoff, P., and Chambers, R., Micrurgical studies in cell physiology. III. The action of CO_2 and some salts of Na, Ca, and K on the protoplasm of *Amœba dubia*, *J. Gen. Physiol.*, 1927, x, 731.

8. Seibert, F. B., personal communication.

EXPLANATION OF PLATE 5.

Fig. 1. A normal ameba. Blur in center indicates rapidity of motion of endoplasm; ectoplasm moving more slowly; distinct plasmalemma.

Fig. 2. Effect of immersion of amebæ in phosphatide emulsion. Lower ameba rounded, plasmalemma quiescent and beginning to disappear, internal cytoplasm active. Upper ameba, plasmalemma dissolved, dead debris left.

Fig. 3. Effect of injection of protein fraction into ameba. Quiescence of internal cytoplasm.
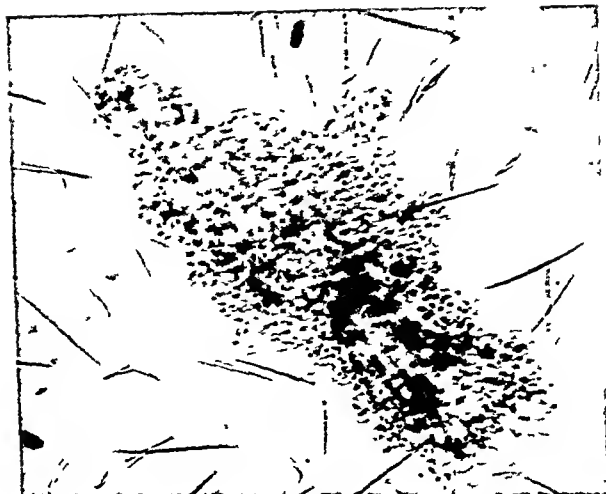
FIG. 1.

FIG 2.

THE RELATION OF STREPTOCOCCI TO HERPES VIRUS ENCEPHALITIS.

By PETER K. OLITSKY, M.D., and PERRIN H. LONG, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

It is generally believed at the present time that no convincing evidence has been brought forward in support of a definite bacterial incitant of epidemic and of herpes virus encephalitis.

Recently, Evans and Freeman[1] have described an amphophilic, aerobic, pleomorphic, green-producing streptococcus which they obtained from nasal washings, the heart blood, and the mesencephalon of a patient who died during an acute attack of epidemic encephalitis. This organism appeared in tubes of chopped meat medium inoculated with the washings, or a few drops of heart blood, or with small pieces of mesencephalon. It grew readily upon ordinary media in subplants and did not ferment salicin, raffinose, mannitol, or inulin. Litmus milk was curdled.

The microorganism grew at various times as a long chain streptococcus, a diplococcus, a spore-bearing rod, a giant coccus, or as a diphtheroid. Of these, the streptococcus was pathogenic in rabbits when inoculated into the brain, and produced a symptom-complex associated with pathological findings which were thought to resemble those of epidemic encephalitis in man.

In a continuation of these studies, Evans[2] reported that she was able to isolate the pleomorphic organism from rabbit brains containing one or another of six different strains of herpes virus. Brains carrying respectively Strains J. B.[3] and H. F.,[4] from The Rockefeller Institute, were among these specimens. Chopped meat medium inoculated with 1 to 2 cc. of a 10 per cent saline suspension of infected brain was used. In three instances the organism was procured directly from glycerolated brain material, while in the case of the other three specimens, it proved necessary for positive cultivation to secure fresh brain material by animal passage. The bacteria were thought to be identical with those isolated by Evans and Freeman from epidemic encephalitis in man.

[1] Evans, A. C., and Freeman, W., *Pub. Health Rep., U. S. P. H.*, 1926, xli, 1095.

[2] Evans, A. C., *Pub. Health Rep., U. S. P. H.*, 1927, xlii, 171.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[4] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

The ease with which the organisms were cultivated by the authors mentioned here led us to a study of their source and their relation to virus encephalitis in the rabbit.   Cultivation experiments have been made with rabbit brains which contained either the J. B.,[3] the H. F.,[4] or the Levaditi[3,5] strains of encephalitogenic virus.

<center>EXPERIMENTAL.</center>

Media.—The following media were used in the cultivation experiments.

Chopped meat medium, prepared according to the directions given by Evans.[2] "Ordinary beef infusion broth is prepared and the hydrogen ion concentration is adjusted to pH 8.0.   Instead of discarding the meat from which the broth is made, the ground meat particles are placed in tubes to a depth of about 1 inch. Sterilization is at 15 pounds for $1\frac{1}{2}$ hours.   During sterilization the hydrogen ion concentration is reduced to about pH 6.8."

Dextrose beef infusion broth containing 1 per cent dextrose.

5 per cent rabbit's blood-beef infusion agar in Petri dishes.

Culture Technique.—Cultures were made not only of brains carrying respectively the three strains of encephalitogenic virus, but of normal brain tissue, of chopped meat particles, and of dextrose broth.   In each test, 1 cc. of a 10 per cent saline suspension of a ground tissue or of broth was inoculated into three to six tubes of fresh medium.   The broth, like the tissue, had been ground as such in a sterile mortar under a hood, with precautions for sterility.

Cultures from Normal and Virus Encephalitis Brain Material.

The results obtained with brain material are summarized in Table I. Cultures were made with herpes virus-infected rabbit brains placed for varying periods of time in sterile 50 per cent glycerol, fresh normal, and fresh virus-infected brains from rabbits and guinea pigs, and fresh brain material of guinea pigs inoculated intracerebrally with non-infective cerebral tissue.

From the table it will be noted that irregular results were obtained. A single culture of *Staphylococcus aureus* was obtained from one of the glycerolated specimens of virus-infected brains, and from the fresh virus-infected brains a streptococcus in one instance and a large coccus in another.   Two strains of streptococci were isolated from normal guinea pig brain material and two from the brain tissue of guinea pigs which had been previously inoculated subdurally with

[5] Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 354; *Ann. Inst. Pasteur*, 1920, xxxiv, 911.

the Levaditi strain of virus. All positive cultures were procured from liquid media, the blood agar plates remaining sterile in all instances.

Cultures of Chopped Meat Medium and of Broth.

It was important to ascertain whether the meat particles used in the medium contained organisms which had resisted sterilization.

TABLE I.

Results of Cultures.

Animal	Virus strain	Status at death	Condition of brain	Growth in chopped meat medium	Growth on blood agar
Rabbit A	Levaditi	Typical virus enceph-alitis	Glycerolated 8 days	*Staphylococcus aureus* in 1 tube	Negative
" B	"	" "	Fresh	Negative	"
" C	"	" "	Glycerolated 11 days	"	"
" D	"	" "	Glycerolated 13 days	"	"
" E	H. F. 1	" "	Glycerolated 90 days	"	"
" F	J. B.	" "	Glycerolated 113 days	"	"
" G	Levaditi	" "	Fresh	Streptococci in 1 of 3 tubes	"
" H	"	" "	"	Large cocci in all tubes	"
Guinea Pig A	H. F. 1	" "	"	Negative	"
" " B	J. B.	" "	"	"	"
" " C	Levaditi	Normal	"	"	"
" " D	"	"	"	Streptococci in 1 of 3 tubes	"
" " E	"	"	"	" "	"
" " F	Control	"	"	Negative	"
" " G	"	"	"	"	"
" " H	"	"	"	Streptococci in 1 of 3 tubes	"
" " I	"	"	"	" "	"

Three uninoculated lots of meat medium, containing 260 tubes, prepared in the usual manner and at different times, were incubated from 3 days to 3 weeks at 37°C. The tubes were examined frequently for bacterial growth and were found to be sterile. As a further check, the meat particles from twelve tubes were smeared upon the surface of blood agar plates. These also remained sterile.

The next experiment concerned the possibility of obtaining organisms from ground meat particles and dextrose broth.

The meat particles from each of twenty-three tubes of uninoculated chopped meat medium were ground in a mortar with the usual precautions against contamination. 1 cc. of the resulting suspensions was inoculated into fresh tubes of meat medium and dextrose broth respectively, and upon blood agar plates. Six tubes of dextrose broth free from growth after 2 weeks incubation, were used to provide control material. From each of these, 4 cc. was removed and "ground" in a sterile mortar. This material was seeded into dextrose broth and upon blood agar plates.

Streptococci were obtained in culture from 6 of the 23 tubes from which the meat particles were removed and ground, diphtheroids from 6, spore-producing rods from 9 (together with streptococci in one case), giant cocci from 14, hay bacilli from 1, and *Staphylococcus albus* from 5. A streptococcus was isolated from one tube seeded with the "ground" dextrose broth. The blood agar plates were sterile.

Description of the Streptococci.

The streptococci procured in the way just mentioned grew readily in chopped meat medium, in dextrose broth, and upon rabbit blood agar plates. The growth was granular in the chopped meat medium and in dextrose broth. Small colonies having a greenish tinge and a small zone of hemolysis appeared on rabbit blood agar plates. Both short and long chain types developed in the liquid media, and often long chains of parallel rows of diplococci. Dextrose, saccharose, maltose, and lactose were fermented, while mannitol, xylose, salicin, inulin, and raffinose were not fermented. The reaction to Gram's stain was variable. The organisms showed no marked pleomorphism, and there was no suggestion of a change to a giant coccus, rod form, or diphtheroid.

Intracerebral Inoculation into Rabbits.

The virulence of eight strains of streptococci isolated as above described was tested by intracerebral injection into rabbits.[6] Two

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[6] All experiments on animals were made with the aid of complete ether anesthesia.

rabbits were used for each strain.   Four of these strains failed to cause death of the rabbits.   Both the animals inoculated with a fifth died of a purulent meningoencephalitis, and so too did one rabbit out of each pair inoculated with the remaining three strains.

The following protocol is typical of the findings in infected animals.

Rabbit A was inoculated intracerebrally with 0.35 cc. of an 18 hour initial dextrose broth subplant of a streptococcus isolated from ground meat particles. 18 hours after injection, the rabbit's temperature rose to 105.2°F.   It was weak, had frequent generalized involuntary muscular contractions, and a Parkinsonian type of head tremor.   The pupils were widely dilated, the breathing slow and labored, and a marked urine retention was present.   The animal became moribund within 24 hours and was etherized.

The brain, removed with sterile precautions, was markedly congested and soft. The cerebrospinal fluid was increased in amount and contained numerous fibrin flakes.   A smear from the brain showed diplococci; and pure cultures of streptococci were isolated from it as also from the heart blood.   Microscopically the brain showed diffuse polymorphonuclear infiltration of the meninges, large hemorrhages and small abscesses in the cortex, generalized edema, and a perivascular infiltration with polymorphonuclear neutrophiles and lymphocytes. In addition, a generalized polymorphonuclear neutrophilic infiltration of the brain tissue was noted.   No intranuclear inclusion bodies were found.

Diagnosis: Purulent meningoencephalitis.

Experiments on Immunity.

Rabbits which had proved resistant to intracranial inoculation of the streptococci were subsequently reinoculated subdurally with active herpes virus.   All died from virus encephalitis.

Six rabbits were injected corneally with an initial culture of streptococci obtained from the brain of a rabbit dying from streptococcic meningoencephalitis.   No keratitis developed, such as is produced by all three of the strains of encephalitogenic virus used in the experiments.   2 weeks later, these six animals were reinoculated in the cornea with the Levaditi strain of virus.   All but one rabbit (an animal discarded from another experiment) developed a characteristic keratitis with fatal termination from virus encephalitis.

No cross-immunity between streptococci and encephalitogenic virus was demonstrated in these experiments.

Differential Glycerolation.

It is not known definitely how long bacteria will survive in glycerolated brain material, kept at +4°C. Herpes virus survives indefinitely under such conditions. The brains of rabbits which had died of herpes virus encephalitis but which contained streptococci, as the cultures showed, were placed in 50 per cent glycerol for 120 and 133 days respectively. At the end of these periods it proved impossible to recover streptococci from the glycerolated brain tissue, in chopped meat medium, dextrose broth, or upon blood agar plates. Yet the same glycerolated brain material, inoculated intracerebrally, produced typical virus encephalitis in rabbits. In the brains of the animals that died no streptococci were found.

SUMMARY AND CONCLUSIONS.

Cultures of microorganisms similar to those described by Evans have been obtained in media inoculated with suspensions of herpes virus-infected brains prepared by grinding. But they have also been isolated from saline suspensions of uninoculated meat particles ground in a sterile mortar, and from dextrose broth treated in the same way. It is believed that these organisms are contaminants introduced during the process of grinding. Since they enter the material in no great number, one may suppose them to be suppressed by animals inoculated with the ground substance. In artificial media, on the other hand, they find favorable conditions for multiplication. In our experience, no growth of microorganisms is obtained in routine cultures of virus-infected brains, when fragments, instead of ground material, are used—a fact which may be taken to support the explanation just given.

The tests of the part played by streptococci in experimental virus encephalitis failed to disclose that the microorganisms have any etiological relationship to the affection. The intracerebral injection of rabbits with the cultures procured in the course of the experiments produces a purulent type of meningoencephalitis which does not resemble virus encephalitis either in its symptom-complex or in its pathology. The same type of meningitis follows the injection of streptococci derived from ground meat particles, from "ground" broth, from normal brains, and those infected with herpes virus. Some

rabbits manifested resistance to the streptococci, whereas all that have been inoculated intracerebrally with the three strains of herpes virus used in this study have proved susceptible thereto. Certain of the rabbits just mentioned which had proved resistant to streptococci inoculated into the brain or cornea were injected with herpes virus and reacted typically. Comparative tests have revealed that the streptococci are more sensitive to the destructive effect of 50 per cent glycerol than is herpes virus. From all this, it can be concluded that streptococci are not the visible form of herpes virus, nor do they produce in rabbits effects like those induced in the brain and cornea by the herpes virus.

STUDIES ON BLOOD CELL METABOLISM.

I. The Effect of Methylene Blue and Other Dyes upon the Oxygen Consumption of Mammalian and Avian Erythrocytes.*

By GEO. A. HARROP, Jr., M.D., and E. S. GUZMAN BARRON, M.D.

(From the Chemical Division of the Medical Clinic of the Johns Hopkins Hospital and University, Baltimore.)

Although a considerable number of studies have been published on various phases of the metabolism of blood cells, the relative importance of fermentation and of respiration in their normal activities is still very imperfectly understood. Still more obscure is the metabolic behavior of pathological blood cells, although it may be of considerable significance. It is of particular interest, for example, to examine the activities of the cells of leucemic blood in the light of the recent advances made in our knowledge of the metabolism of cancer cells.

A question which at once attracts attention in this field is that of the cause of the great variations which are found in the respiration of various types of normal erythrocytes. Several years ago it was shown by one of us (1) that normal human erythrocytes, separated from leucocytes and blood platelets by appropriate methods, have a scarcely demonstrable oxygen consumption when incubated at 38°C. for some hours under sterile conditions. The utilization of oxygen was considerable, however, when large numbers of reticulocytes were present in the blood, such as are found in hemolytic jaundice or in other conditions with active blood regeneration. These findings in general were in harmony with the previous studies of Morawitz and Itami (2) upon the oxygen consumption of anemic blood, and of Warburg (3), who found, using the Barcroft-Haldane manometer, that although normal adult mammalian erythrocytes in general had a very low oxygen consumption, the respiration was much increased in the blood of

* The second paper of this series will appear in a forthcoming number of *The Journal of Biological Chemistry.*

young rabbits and appeared to be roughly proportional to the amount of polychromasia present.   In contrast to the slight oxygen consumption of non-nucleated mammalian erythrocytes, Warburg showed that the nucleated erythrocytes of birds (geese) have a very large oxygen consumption.

By the use of the technique previously described (1), but with the more accurate Van Slyke constant volume blood gas apparatus now available, it can be shown that non-nucleated adult mammalian erythrocytes have an exceedingly small, but measurable oxygen consumption when incubated at 38°C.   This is accompanied by the production of carbon dioxide.

The interesting question now arises as to the cause of this difference in the metabolism of mature mammalian erythrocytes, in contrast to that of the young or immature forms and that of the nucleated cells of avian blood.   To state that the oxidative activities of the different types of red blood cells seem to be related in some intimate way to the presence or absence of nuclear material furnishes no satisfactory clue as to the actual mechanism involved.   It is conceivable that in the course of its development some inhibiting substance is formed in the mammalian erythrocyte which almost stops the respiratory metabolism of the cell.   On the other hand, it is also conceivable that in the course of development, some essential link in the respiratory mechanism is lost in the cell so that oxidations no longer take place.   We wish to present evidence for the latter view.   We have found that the oxygen consumption of non-nucleated mammalian erythrocytes can be greatly increased, and can approach the magnitude of that of the nucleated cells of avian (goose) blood when one of a number of vital dyes, and particularly methylene blue in low concentration (0.005 to 0.0005 per cent), is added to the blood before its incubation.* The amount of oxygen absorption when compared to that in ordinary defibrinated blood may be increased many times.   This rise occurs in the blood of all mammals tested—dog, beef, sheep, and man, and is accompanied by a corresponding evolution of carbon dioxide (Table I).

* The methylene blue used in these experiments was Purified Methylene Blue from Leopold Cassella and Co., Frankfurt a.M.

TABLE I.

The Effect of Methylene Blue upon the Respiration of Mammalian Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed
	vol. %	*vol.* %	*vol.* %	*vol.* %	*per cent*
1. Man—defibrinated blood					
Control before incubation..................	36.79	19.24			
after incubation....................	38.57	18.94	1.78	0.30	1.6
+M.B. after incubation....................	48.01	6.82	11.22	12.42	64.6
2. Man—washed cells suspended in Locke's solution					
Control before incubation..................	4.22	17.01			
after incubation....................	4.57	16.53	0.35	0.48	2.8
+M. B. after incubation....................	14.64	3.39	10.42	13.62	80.1
3. Dog—defibrinated blood					
Control before incubation..................	13.91	23.20			
after incubation....................	19.45	22.77	5.54	0.43	1.9
+M.B. after incubation....................	22.17	12.64	8.26	10.56	45.5
4. Dog—defibrinated blood					
Control before incubation..................	9.99	27.95			
after incubation....................	19.35	27.80	9.36	0.15	0.05
+M.B. after incubation....................	25.00	14.40	15.01	13.55	48.5
5. Sheep—defibrinated blood					
Control before incubation..................	21.64	16.32			
after incubation....................	26.92	16.10	5.28	0.22	1.5
+M.B. after incubation....................	32.17	7.94	10.53	8.38	51.4
6. Beef—defibrinated blood					
Control before incubation..................		22.62			
after incubation....................		22.03		0.59	2.6
+M.B. after incubation....................		12.84		9.78	43.3
7. Dog*—defibrinated blood					
Control before incubation..................	13.59	22.01			
after incubation....................	15.74	20.02	2.15	1.99	9.2
+M.B. after incubation....................	21.35	10.09	7.76	11.92	54.1

The incubation time in all of these experiments was 3 hours at 37°.   Methylene blue (M.B.) 0.005 per cent.

* Blood taken from a dog that had been previously subject to large bleedings. Examination of the blood for immature forms was not made.

TABLE I—*Concluded*.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen con-sumption	Oxygen con-sumed
	vol. %	*vol. %*	*vol. %*	*vol. %*	*per cent*
8a. Man—defibrinated blood					
Control before incubation..................	11.11	20.45			
after incubation.....................	16.47	19.47	5.36	0.98	4.8
+M.B. after incubation...................	25.60	11.32	14.49	9.13	44.6
8b. Man—washed cells of above (8a) suspended in Locke's solution					
Control before incubation..................	5.28	19.95			
after incubation.....................	5.92	19.24	0.64	0.71	3.6
+M.B. after incubation...................	12.80	10.93	7.52	9.02	45.2
9. Man†—washed cells suspended in Locke's solution					
Control before incubation..................	2.94	22.95			
after incubation.....................	5.30	20.35	2.36	2.60	11.3
+M.B. after incubation................:....	13.68	8.44	10.74	14.51	63.4
10. Dog—washed cells suspended in Locke's solution					
Control before incubation..................	3.36	22.58			
after incubation.....................	4.35	21.62	0.99	0.96	4.3
+M.B. after incubation...................	13.56	9.58	10.20	13.00	57.6

† Blood from a patient with polycythemia vera. A good deal of polychromasia and some immature cells present. It may be seen that in this experiment the percentage acceleration of respiration on the addition of methylene blue is much less than with normal human erythrocytes.

The blood used in these studies was freshly drawn and carefully defibrinated with glass beads or a stirring rod. It was then passed with very gentle suction once or twice through a column of several centimeters of sterile cotton wool. The filtration was carried out in the cold (10°C.) to prevent glycolysis. This proved a most effective method of removing the leucocytes, as demonstrated by numerous control counts, which rarely exceeded 1000 per c. mm. The blood was then thoroughly saturated with oxygen and a control sample taken for immediate gas analysis. A portion was then treated with the dye or other reagents whose effect was to be studied, and another portion, untreated, was taken for a control. These samples were incubated at once either under a thick layer of paraffin oil, or, when carbon dioxide analyses were also desired, in small stoppered bottles, provided with a long side arm capillary, essentially as described in the earlier paper(1). They contained a few glass beads and were well paraffined. Certain control analyses were also done by means of a microrespiration apparatus.

The microrespiration apparatus used was slightly modified from the design of

that described by Winterstein (4). Our vessels were made in the shape of tiny Erlenmeyer flasks with very broad bases. Two small chambers were blown out from the vessel wall at opposite sides to carry other fluids, whose effect on the respiration it was desired to test. In the present instance these were methylene
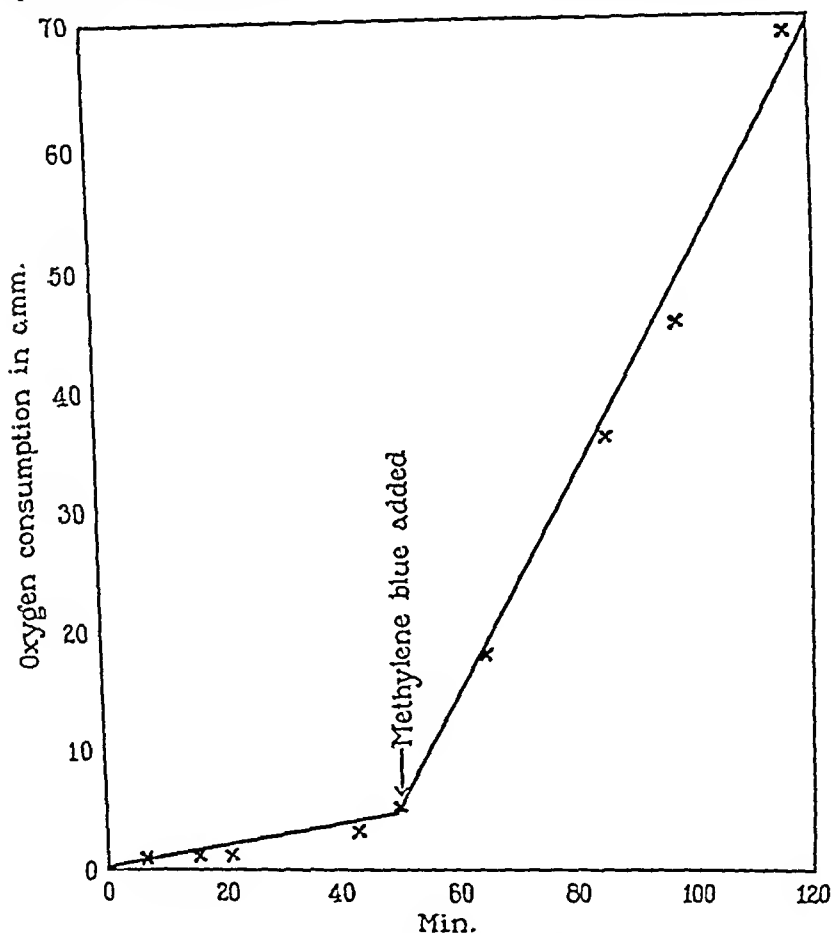
CHART 1. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of human erythrocytes (1.0 cc.).

blue or other dyes, and potassium cyanide, or other agents which inhibit cell oxidations. A special shaking device was constructed, and the apparatus was shaken at a rate of 75–100 per minute. A large rubber bulb with set screw attachment was used to regulate the mercury burette level. When two essential

precautions were observed—absolute cleanliness of the capillary tubes which contained the bubble, and care in the maintenance of a constant temperature of the water bath—the apparatus proved very useful in our hands.   It presents certain advantages over the Barcroft-Warburg method in that elaborate calibration and calculations are avoided, since the oxygen consumption is found by direct readings on the mercury burette.

The increased oxygen consumption of human erythrocytes upon the addition of methylene blue in the microrespiration apparatus is indicated in the experiment shown in Chart 1.

We have excluded the possibility of methemoglobin formation in these studies by spectroscopic examination and particularly by a

TABLE II.

The Effect of Temperature upon the Oxygen Consumption of Mammalian (Dog) Erythrocytes.

	Oxygen content before incubation	Oxygen content after 3 hrs. incubation	Oxygen content after 24 hrs. incubation
	vol. %	*vol. %*	*vol. %*
Control at 21°C........................	19.45	19.20	18.84
+M.B. 0.05%...........................		17.40	13.92
Control at 38°C		18.80	12.72
+M.B. 0.05%...........................		9.60	Completely reduced

Methylene blue (M.B.) concentration 0.005 per cent.

second estimation of the oxygen capacity of the blood after incubation, upon resaturation with air or oxygen.   The latter is the more reliable test.   Methylene blue alone gives a prominent line between the characteristic bands of methemoglobin in the spectroscope which may readily cause confusion on casual inspection.

The recent study of Wales, Munch and Schwartze (5) is of interest in regard to the question of methemoglobin formation.   The authors made intravenous injections into rabbits and cats of very large amounts of certain dyes used for the coloring of foods and then made spectrophotometric determinations upon blood samples withdrawn at various intervals.   With all of the dyes studied, reduced hemoglobin was formed within an hour.   Complete reduction occurred when the specimens were permitted to stand for 24 hours or more.   No evidences of methe-

moglobin formation could be detected in any of their studies. It seems likely that these dyes produced an effect similar to that of methylene blue upon the respiration of the erythrocytes.

The respiration takes place in fresh red blood cells carefully washed with Ringer's solution at approximately the same rate as in defibrinated blood alone and the increased respiration on addition of methylene blue is of about the same order of magnitude for both as is indicated in Experiments 8a and 8b, Table I.

The accelerated oxygen consumption in the presence of methylene blue proceeds even at room temperature, but its optimum appears to be at 37°C. (Table II).

TABLE III.

The Effect of Varying the Methylene Blue Concentration upon the Respiration of Mammalian Erythrocytes.

Nov. 21—dog blood	Before incubation		After incubation		Output carbon dioxide	Consumption oxygen
	Content carbon dioxide	Content oxygen	Content carbon dioxide	Content oxygen		
	vol. %	*vol. %*	*vol. %*	*vol. %*	*vol. %*	*vol. %*
Control—no M.B.	17.75	29.25	19.86	28.93	2.11	0.32
M.B. 0.005%			29.88	14.45	12.13	14.80
M.B. 0.0025%			30.00	14.21	12.25	15.04
M.B. 0.001%			28.75	14.80	11.00	14.45
M.B. 0.0005%			27.98	18.06	10.23	10.19

Methylene blue (M.B.) concentration 0.005 per cent.

The final concentration of methylene blue used in these studies has been 0.005–0.0005 per cent. Higher concentrations produced no marked increase in the respiration and at 0.0005 per cent concentration there was a diminution in the effect of the added methylene blue (Table III).

It is known that potassium cyanide, even in very dilute solution, stops cell oxidations. We have therefore studied the effect of potassium cyanide upon methylene blue respiration in the experiments presented in Table IV. It will be seen that the increased oxygen consumption upon the addition of methylene blue was nearly as great in the presence of the cyanide as in the untreated control. The effect

of methylene blue upon the respiration of mammalian blood cells is not therefore inhibited by this concentration of potassium cyanide (N 0.001).

The effect of methylene blue upon the active respiration of the nucleated red blood cells of birds was found to be entirely different from the effect which it had on the non-nucleated mammalian red blood cells.   As is indicated in Table V and in the microrespiration experi-

TABLE IV.

Effect of Potassium Cyanide upon Methylene Blue Respiration.

	Content oxygen		Content oxygen
	vol. %		*vol. %*
Beef			
Blood before incubation..............	22.61	After 3 hrs. incubation	22.03
Blood + N/5000 KCN................		After 3 hrs. incubation	21.95
Blood + 0.005% methylene blue......		After 3 hrs. incubation	12.84
Blood + N/5000 KCN + 0.005% M.B.............................		After 3 hrs. incubation	12.74
Dog			
Blood before incubation..............	23.80	After 3 hrs. incubation	23.40
+ N/5000 KCN................		After 3 hrs. incubation	23.58
+ 0.005% methylene blue......		After 3 hrs. incubation	14.63
+ N/5000 KCN + 0.005% M.B.......................		After 3 hrs. incubation	15.10
Man			
Blood before incubation..............	19.42	After 3 hrs. incubation	19.18
+ N/1000 KCN................		After 3 hrs. incubation	19.09
+ 0.005% methylene blue......		After 3 hrs. incubation	9.42
+ N/1000 KCN + 0.005% M.B.......................		After 3 hrs. incubation	11.70

ment shown in Chart 2, the respiration of goose erythrocytes, either from normal blood or from animals rendered anemic by bleeding, is increased by methylene blue, but to a much smaller extent than in the case of non-nucleated mammalian cells.   Whereas in the latter the respiration is accelerated by methylene blue on the average twenty to fiftyfold, in avian blood it is never increased more than twice that of the simple defibrinated blood to which dye has been added.

Chart 3 illustrates the results obtained from a microrespiration experiment and shows that the respiration of goose erythrocytes which

TABLE V.

The Effect of Methylene Blue upon the Respiration of Avian (Goose) Erythrocytes.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed	Time of incubation	Condition of animal
	vol. %	vol. %	vol. %	vol. %	per cent		
1. Dec. 6—defibrinated blood							
Control before incubation....	11.59	21.60					
after incubation......	18.77	11.63	7.18	9.97	46.2	3 hrs.	Normal
+M.B. after incubation......	30.98	1.01	19.39	20.59	95.5		
2. Dec. 16—defibrinated blood							
Control before incubation....	12.00	16.55					
after incubation......	20.46	7.84	8.46	8.71	52.6	1½	Anemic
+M.B. after incubation......	24.28	1.36	12.28	15.19	91.6	hrs.	
+T.B. after incubation.......	23.51	1.76	11.51	14.79			
3. Feb. 1—defibrinated blood							
Control before incubation....	22.64	11.24					
after incubation......	26.20	7.05	3.56	4.19	37.2	40	Anemic
+M.B. after incubation......	28.42	5.03	4.78	6.21	55.2	min.	
4. Feb. 6—defibrinated blood							
Control before incubation....	22.40	22.00					
after incubation......	32.05	16.92	9.65	5.08	23.1	40	Normal
+M.B. after incubation......	35 50	14.16	13.10	7.84	35.6	min.	

The blood for Experiments 1, 2, and 3 was taken from the same animal during the course of several large periodical bleedings.

	Content carbon dioxide	Content oxygen	Carbon dioxide output	Oxygen consumption	Oxygen consumed	Time of incubation	Condition of animal
5. Dec. 21—washed cells in Locke's solution							
Control before incubation....	4.00	19.65					
after incubation......	10 66	11.35	6.66	8 30	42.2		
+M.B. after incubation ...	12 66	8 44	8 66	11.21	57.1		Anemic

M.B. means methylene blue 0.005 per cent solution.

T.B. means toluylene blue 0.005 per cent solution.

has been inhibited by the addition of potassium cyanide may be again restored when methylene blue is added.

We have extended our studies to an examination of the effect of·

several other dyes whose oxidation-reduction potentials have been determined, including Bindschedler's green, indigo disulfonate, *o*-chlorophenol indophenol, phenol indophenol, and toluylene blue. Some
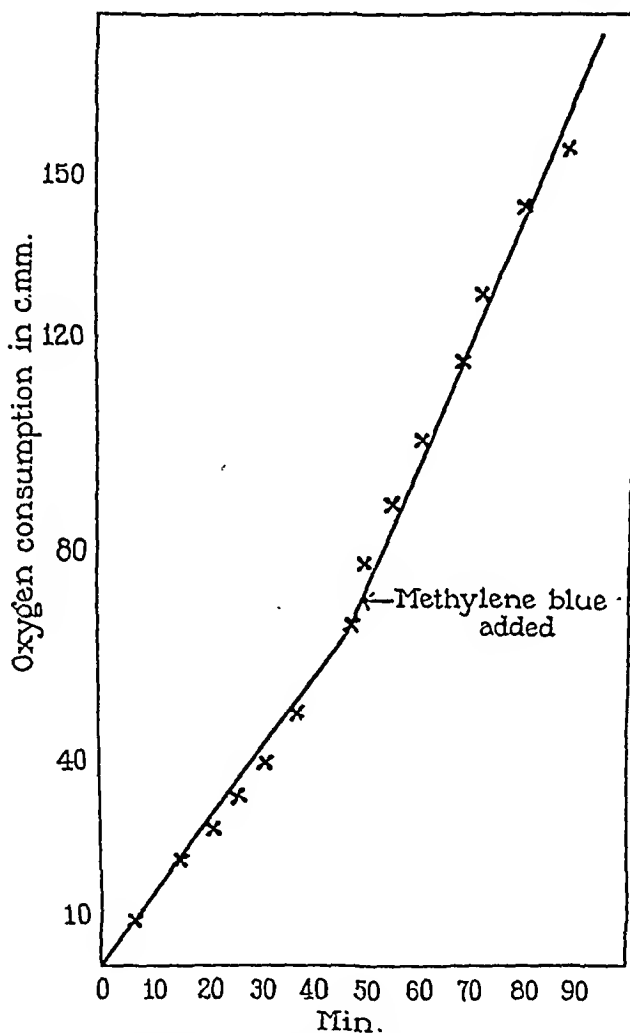
CHART 2. Microrespiration experiment. The effect of methylene blue upon the oxygen consumption of goose erythrocytes.

of these were kindly supplied to us by Professor W. Mansfield Clark. As seen in Table V, Experiment 2, and in Table VI, none of these with the exception of toluylene blue is as effective as methylene blue in

stimulating respiration at this range of concentration. The fact that no direct correlation can be shown between the oxidation-reduction potential of these dyes and the kinetic phenomena involved in the
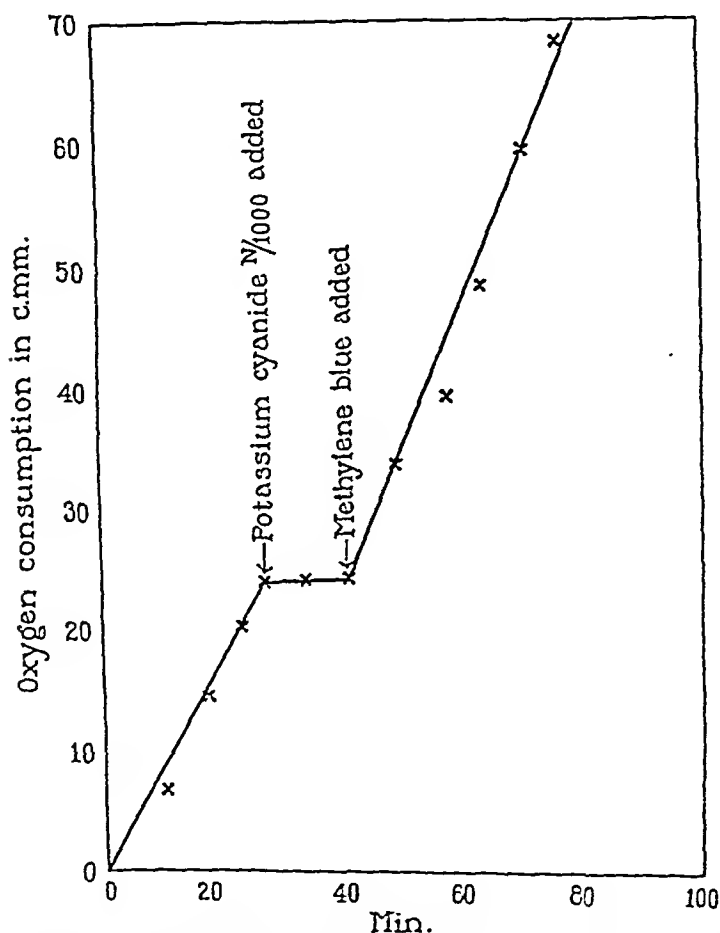
CHART 3. Microrespiration. The effect of methylene blue upon the oxygen consumption of goose erythrocytes in the presence of potassium cyanide.

accelerated cellular oxidations is not surprising. The question of the variable permeability of the cell membranes to the several dyes and the possibility of their variable toxic action must be taken into considera-

tion.   It is at least of some significance that the potentials of the dyes which activate these oxidations fall within a rather restricted range.

The increased red blood cell metabolism reported in the present paper recalls the work of Heymans and Heymans (6), who produced fever in dogs ranging as high as 43° or 44°C., together with a marked increase in the output of carbon dioxide, by the intravenous injection of methylene blue, 0.05–0.10 per kilo of body weight.

TABLE VI.

The Effect of Various Dyes upon the Respiration of Mammalian Erythrocytes.

Oct. 28—beef blood; 3 hrs. incubation	Oxygen content before incubation (A)	Oxygen content after incubation (3 hrs.) 38°C.) (B)	Oxygen consumption (A–B)	Percentage oxygen consumption $\frac{A-B}{A} \times 100\%$
	vol. %	*vol. %*	*vol. %*	
Control blood alone..........................	18.50	18.11	0.39	2.1
Methylene blue 5 mg. % added....................		11.70	6.80	36.8
Indigo disulfonate 5 mg. % added..................		17.75	0.75	4.1
o-Chlorophenol indophenol 5 mg. % added..........		16.45	2.05	11.1
Phenol indophenol 5 mg. % added.................		16.28	2.22	12.0
Toluylene blue 5 mg. % added....................		10.00	8.50	46.0
Dec. 21—goose blood; 1 hr.'s incubation				
Control blood alone..........................	19.65	11.35	8.30	42.2
Methylene blue 0.0025%.........................		8.44	11.21	57.1
Bindschedler's green 0.0025%......................		9.04	10.61	54.0
Dec. 22—human blood (polycythemia vera); 3 hrs. incubation				
Control blood alone..........................	23.58	22.35	1.23	5.2
Methylene blue 0.0025%.........................		8.44	15.14	64.4
Bindschedler's green 0.0025%......................		15.52	8.06	34.2

In seeking an explanation of the effect of methylene blue upon erythrocyte respiration our attention was drawn to the studies reported by Meyerhof (7) upon staphylococci, precipitated by acetone in the cold, dried over phosphorus pentoxide, and subsequently heated *in vacuo* at 100–107°C.   Under such circumstances, and by proper selection of the heating time, he was able to suppress to a large extent the oxygen absorption of this material.   Upon the addition of methyl-

ene blue, 2.5 or 5 mg. per cent, the oxygen utilization again increased, although never to the same extent as in the unheated material. The methylene blue, by reason of its oxygen carrying properties was considered to take the place of some component of the respiration system which had been destroyed by the previous manipulations. The usual inhibiting effect of potassium cyanide upon the oxygen consumption of the killed staphylococci was much less marked in the presence of the methylene blue. The respiratory quotient lay within physiological limits -0.6 to 1.0.

Meyerhof reports a similar effect upon the oxygen consumption of acetone yeast, although here the optimal concentration of methylene blue was higher $(0.02 - 0.1$ mg. per cent). It was found to be unnecessary to heat the acetone yeast because the respiration of the unheated yeast, after the acetone-ether treatment, had already fallen to about 2 per cent. The respiration of the living cells, which are much less affected by methylene blue, was not inhibited in the presence of 0.0015 N KCN when 0.05 per cent methylene blue was added. Acetone yeast respiration in the presence of methylene blue is only affected to the extent of 10–20 per cent even in the presence of 0.01 N KCN. Macerated yeast juice (Lebedew) was found to behave in a similar manner in the presence of methylene blue.

The oxidation mechanism in the experiments which we report in the present paper appears to be analogous to that encountered by Meyerhof in his experiments. The effect of methylene blue occurs even in the presence of cyanides in both sets of experiments. The rate of oxidation in the mammalian erythrocytes in the presence of methylene blue is increased to about the rate of oxygen consumption found in the untreated nucleated erythrocytes of birds. Mammalian erythrocytes can be regarded as old or altered cells, in which oxidation processes have been largely suppressed through the disappearance of some link of the oxidation system. This link, however, is present in the younger cells. Methylene blue appears to be able to take the place of this substance and to act essentially as an oxygen carrier enabling the erythrocytes to utilize in their metabolism the oxygen stored in them as oxyhemoglobin. The respiration then resumes a rate similar to that present in the nucleated erythrocytes of avian blood.

The demonstration of proper respiratory quotients in studies on the

metabolism of blood has been difficult. Most authors content themselves with the statement that "physiological" respiratory quotients are obtainable, but actual figures are seldom given. At the time of our previous paper (1) we were unable to obtain agreement in this regard between oxygen consumption and carbon dioxide production in human blood. Consequently no figures on the carbon dioxide

TABLE VII.

The Respiratory Quotient of Mammalian and Avian (Goose) Erythrocytes with and without the Presence of Methylene Blue. Washed Cells Suspended in Locke's Solution.

	Content carbon dioxide (A)	Content oxygen (B)	Carbon dioxide output (C)	Oxygen consumption (D)	Respiratory quotient $\frac{C}{D}$
	vol. %	vol. %	vol. %	vol. %	
*Man**					
Control before incubation...............	4.22	17.01			
after incubation..................	4.57	16.53	0.35	0.48	0.73
+M.B. after incubation...................	14.64	3.39	10.42	13.62	0.77
Dog†					
Control before incubation...............	3.36	22.58			
after incubation..................	4.35	21.62	0.99	0.96	1.00
+M.B. after incubation...................	13.56	9.58	10.20	13.00	0.79
Goose‡					
Control before incubation...............	4.00	19.65			
after incubation..................	10.66	11.35	6.66	8.30	0.80
+M.B. after incubation...................	12.66	8.44	8.66	11.21	0.78

M.B. means methylene blue 0.005 per cent.
* Experiment 2 Table I.
† Experiment 10 Table I.
‡ Experiment 5 Table V.

production were published. The difficulty in metabolism studies upon incubated mammalian erythrocytes lies in the excessive production of carbon dioxide compared to the oxygen consumption, as may readily be observed by an inspection of Table I. A large initial formation of carbon dioxide in shed blood appears to occur in the serum in the presence of the red cells. It does not occur when the cells are

washed and suspended in Locke's solution (Table I, Experiments 8a and 8b). The amount of extra carbon dioxide thus produced in the 1st hour of incubation may amount to 2 millimols per 100 cc. of blood. The source of this extra carbon dioxide is under investigation. As seen from Table VII, the respiratory quotient of both avian and mammalian erythrocytes usually lies between 0.75 and 0.80 both in the presence of methylene blue and in its absence, when the serum is removed and the cells are suspended in Locke's solution.

TABLE VIII.

Effect of Hemolysis (Freezing and Thawing) upon Oxygen Consumption of Goose Blood.

	Oxygen content before incubation	Oxygen content after incubation	Oxygen consumption
	vol. %	vol. %	%
Control blood..............................	16.55	7.84	52.6⎫ 1 hr.
Control blood + 0.005% M.B.................		1.76	89.4⎭ incubation

After hemolysis by freezing and thawing, the material was centrifuged at high speed and the top and bottom layers separated. The top material contained only a small amount of cellular debris and no nuclear fragments when examined microscopically. The bottom layer contained cell fragments and nuclei and a very few intact erythrocytes.

Top layer control............................	10.84	10.40	4.0⎫ 2 hrs.
Top layer control + 0.005% M.B..............		9.13	15.8⎭ incubation
Bottom layer control	13.67	10.90	20.3⎫ 2 hrs.
Bottom layer control + 0.005% M.B...........		7.94	41.9⎭ incubation

Effect of Destruction of the Cell Membrane (Freezing and Thawing) upon Oxygen Consumption and the Effect of Methylene Blue.

Warburg has shown that the cell membranes of erythrocytes (goose) can be destroyed by freezing and thawing. When the material is then centrifuged at high speed, two layers may be obtained, the top containing only hemolyzed cell contents, while the lower contains nuclear material and the remains of the formed elements. He found that the respiratory activity was then confined to the lower layer which contained the nuclear material. We have repeated these experiments,

and have found (Table VIII) that the oxygen consumption is greatly reduced in both top and bottom layers, as compared to the consumption in untreated defibrinated blood. Although the oxygen consumption of the material in the top layer is much less than it is in the bottom layer, it is still appreciable. The addition of methylene blue produces the usual increase in oxygen consumption in both layers, but it is interesting to note that the effect is much more marked in the very slowly respiring top layer which contains no nuclear material.

CONCLUSIONS.

1. The respiratory metabolism of non-nucleated mammalian erythrocytes is enormously accelerated and approaches the magnitude of the metabolism of the nucleated erythrocytes of birds on the addition of methylene blue (and certain other dyes), to a final concentration of 0.005–0.0005 per cent.

2. In the presence of methylene blue the respiration is accelerated even when $M/1000$ KCN is also present.

3. The accelerated respiration due to methylene blue occurs at room temperature but it is most active at 38°.

4. Methylene blue in the above concentration accelerates the respiration of avian (goose) erythrocytes to a much smaller extent than it does the respiration of the erythrocytes of mammalian blood, while the effect upon anemic goose blood seems to be less than it is upon cells of normal goose blood.

5. Owing to a rather large initial carbon dioxide formation in defibrinated blood on incubation, which may not be related to the immediate respiratory process, proper respiratory quotients cannot be obtained in whole blood. When the cells are separated from the serum and suspended in Locke's solution, respiratory quotients are obtained upon incubation comparable to those of other resting mammalian cells, as well as of the actively respiring erythrocytes of birds.

6. The hypothesis is advanced that methylene blue acts in the rôle of an oxygen carrier, supplying a substance which has disappeared from adult mammalian non-nucleated erythrocytes and restoring their metabolic activity to an extent comparable to that of the young immature forms, or to that of the actively respiring avian (goose) blood.

We wish to acknowledge the kindness of Professor Michaelis in drawing our attention to the analogy between our findings and those reported by Meyerhof.

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IMMUNOLOGICAL STUDIES IN RELATION TO THE SUPRA-RENAL GLAND.

III. The Effect of Injections of Epinephrine on the Hemolysin Formation in Normal Rats.

By J. MARMORSTON-GOTTESMAN, M.D., and DAVID PERLA, M.D.

(From the Division of Laboratories, Montefiore Hospital, New York.)

In the previous studies on normal and suprarenalectomized rats, it was found that suprarenalectomy results in an early depression in hemolysin forming capacity during several weeks following the operation (1, 2). Traumatization of the perisuprarenal tissue had the same effect on hemolysin formation as suprarenalectomy. In traumatizing the perisuprarenal tissue the nerve and vascular supply of the suprarenal gland are undoubtedly severely injured. It has been thoroughly established by the work of Dreyer (3), Tscheboksaroff (4), and Stewart and Rogoff (5) and others that the output of epinephrine from the suprarenal gland is controlled by a nervous mechanism, and that interruption of the nervous pathway results in a diminution of the epinephrine output. In this connection, it is of interest to determine the influence of epinephrine injections on the antibody formation in normal and suprarenalectomized rats.

Josue and Paillard (6) found that injections of epinephrine had no influence on opsonin formation. Hrma (7) was unable to observe an effect from injections of epinephrine on the hemolysin titer or complement content of the serum of rabbits though Pinner (8) noted a decrease in the complement titer of guinea pig serum. Bijlsma (9) did not succeed in varying the agglutinin or hemolysin forming capacity of normal and unilaterally suprarenalectomized rabbits by epinephrine injections. Borchardt (10) noted an increase in typhoid agglutinins in human beings following the injection of epinephrine subcutaneously administered 20 days after the injection of the typhoid vaccine. Oda (11) likewise observed an acceleration of the typhoid agglutinin formation following injections of epinephrine in human beings. The bactericidal action of serum of rabbits following the subcutaneous or intraperitoneal injections of epinephrine is increased. Large doses of epinephrine, however, exert an opposite effect (12).

`Method.`

In all the experiments recorded Mulford's "adrenin" was used (1/1000). Dilutions were made in physiological saline and all injections were administered subcutaneously. The daily amounts used for each rat were brought up to a total volume of 1 cc. with physiological saline and this quantity was administered in two injections of 0.5 cc. each at intervals of 6 hours. All the rats used in the experiments recorded in this communication were normal adult albino rats approximately 4 months old. Titers were determined 5, 8, 11, and 14 days subsequent to the injection of the antigen. The antigen employed in all instances was a single intraperitoneal injection of 1 cc. of a 10 per cent suspension of sheep cells.

In determining the titer of hemolysin present in the serum, progressive dilutions of the rat serum to be tested were made. .1 cc. of a 5 per cent suspension of sheep cells and $2\frac{1}{2}$ units of complement were added. The total volume of each tube was brought up to 1 cc. with physiological saline. The tubes were incubated at 37° during 30 minutes and readings were made immediately. Traces of hemolysis equivalent to a one plus or lesser reading on a scale in which four plus represents complete hemolysis, were disregarded. That dilution in which hemolysis was partial but definite, that is, a two plus reaction, was read as the titer of the hemolysin present in the serum.

In the first group of experiments the daily quantity of adrenin injected was kept constant but the number of days that the adrenin was administered prior to and subsequent to the injection of the sheep cells varied. Thus three series were used in this group. All rats in this group received 0.4 mg. of adrenin per kilo per day in two doses. In all instances a 24 hour period was allowed to intervene before and after the injection of sheep cells during which period no adrenin was given. This was done to eliminate any possible immediate influence of epinephrine on the absorption of antigen into the circulation. The first series was given injections of adrenin during 3 days prior and 4 days subsequent to the injection of the antigen. The second series received the injections only during 4 days subsequent to the injection of antigen. The third series of the first group was injected only during 1 day prior and 2 days subsequent to the injection of sheep cells.

In the second group of experiments, the number of injections was kept constant but the daily quantity injected was varied. Thus all rats in this group received injections of adrenin during 3 days prior and 4 days subsequent to the injection of sheep cells. One series, however, received 0.08 mg. per kilo per day or one-fifth the quantity employed in the first group of experiments. A second series received daily injections of 0.02 mg. per kilo per day or one-twentieth the quantity. A third series received 0.01 mg. or one-fortieth, a fourth series, 0.006 mg. or one-sixtieth the amount used in the first group of experiments.

In all series control rats were injected with equivalent volumes of physiological saline in two daily injections. Hemolysin formation in normal rats was also studied.

TABLE I.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injections of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total* daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	mg.						
256	260	0.1	7	14	800	Died		
257	220	"	"	"	0	0	0	
258	240	"	"	"	80	800	400	
259	190	"	"	"	30	200	160	60
260	195	"	"	"	200	300	200	100
261	280	"	"	"	0	200	40	
290	250	"	"	"	160	300	300	100
291	215	"	"	"	600	300	300	160
292	200	"	"	"	160	160	300	160
293	290	"	"	"	400	Died		
294	280	"	"	"	1,600	800	1,600	100
296	190	"	"	"	60	80	Died	
360	150	0.06	"	"	400	360?	80	
361	140	"	"	"	80	300	Died	
362	160	"	"	"	150	400	Died	
363	170	"	"	"	400	800	0	
364	190	"	"	"	4,000	2,000	60	
365	190	"	"	"	800	600	0	
			Saline controls					
397	240	1 cc.	"	"	6,000	Died		
262	190	"	"	"	4,000	400	Died	
356	220	"	"	"	6,000	3,000	200	200
398	190	"	"	"	12,000	4,000	160	
298	230	"	"	"	12,000	2,000	1,000	80
299	200	"	"	"	6,000	1,000	1,000	200
366	225	"	"	"	1,600	800	80	
367	230	"	"	"	4,000	4,000	100	
			Normal rat					
297	210	—	—	—	12,000	4,000	4,000	400
295		—	—	—	2,000	300	300	200

* Total daily volume brought up to 1 cc. with physiological saline.

TABLE II.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily. nmt. of ndrenin	No. of days ndrenin injected	No of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
340	140	0.06	4	8	200	—	100	200
341	155	"	"	"	40	40	40	40
342	155	"	"	"	400	160	20	40
343	140	"	"	"	400	160	60	20
344	170	"	"	"	1,600	400	300	200
345	160	"	"	"	3,000	1,600	600	—?
			Soline controls					
346	140	1 cc.	"	"	4,000	400	80	80
347	140	"	"	"	400	80	0	0
			Normal rats					
264	200	—	—	—	6,000	1,600	300	60
265	210	—	—	—	18,000	6,000	2,000	200

TABLE III.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.4 Mg. per Kilo per Day in 2 Doses, Administered during 1 Day Prior and 2 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
330	150	0.06	3	6	16,000	3,000	600	200
331	145	"	"	"	16,000	1,600	200	160
332	145	"	"	"	8,000	1,000	200	200
333	160	"	"	"	16,000	3,000	400	200
334	140	"	"	"	8,000	600	100	80
335	150	"	"	"	4,000	600	60	20
			Saline controls					
336	140	1 cc.	"	"	32,000	4,000	800	300
			Normal controls					
338	140	—	—	—	32,000	4,000	1,000	200
339	150	—	—	—	2,000	100	10	10

I. The Effect on Hemolysin Formation of Subcutaneous Injections of Epinephrine in Amounts of 0.4 Mg. per Kilo per Day.

In the first series the epinephrine was administered during 3 days prior and 4 days subsequent to the injection of sheep cells. In this series 28 rats were used, of which 18 were treated with epinephrine, 8 were saline controls, and 2 normal controls. The hemolysin formation in the epinephrine treated rats was markedly depressed in 17 of the

TABLE IV.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.8 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
					5th day	8th day	11th day	14th day
	gm.	*mg.*						
350	260	0 02	7	14	6,000	8,000	400	400
351	255	"	"	"	2,000	2,000	200	Died
352	250	"	"	"	800	800	160	160
353	260	"	"	"	800	600	160	30
354	255	"	"	"	800	1,000	60	20
355	258	"	"	"	600	200	40	160
			Saline control					
356	255	1 cc.	"	"	6,000	3,000	200	200
			Normal control					
359	258	—	—	—	2,000	—	300	200

18 rats. One gave a normal titer. The average initial titer in these rats* was 1/328 as compared to the average titer of normal rats of 1/7000. The saline control rats gave normal titers. This depression was strikingly consistent throughout the experiment. The most marked depression was noted in this series.

In the second series the same daily quantity of epinephrine was given during 4 days subsequent to the injection of the antigen. Ten rats were used, of which 6 were treated with epinephrine, 2 were saline controls, and 2 normal rats. The average titer of the treated

* A single normal reading of 1/4000 was omitted in this average. Including this exception, the average titer of the series is 1/551.

rats was 1/940 or still definitely lower than the normal or saline control rats.

The third series of the first group received the same large daily injections of epinephrine but only during 1 day prior and 2 days subsequent to the injection of the sheep cells.   Nine rats were used, of which 6 were injected with epinephrine, 1 with saline solution, and 2 were normal rats.   All gave high normal titers.

TABLE V.

Effect on the Hemolysin Formation in Normal Rats of Subcutaneous Injections of Adrenin in Amounts of 0.02 Mg., 0.01 Mg., and 0.006 Mg. per Kilo per Day in 2 Doses, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of 1 Cc. of a 10 Per Cent Suspension of Sheep Cells.

Rat No.	Weight	Amt. of adrenin per kg.	Total daily amt. of adrenin	No. of days adrenin injected	No. of injections adrenin	Titer			
						5th day	8th day	11th day	14th day
	gm.	*ms.*	*ms.*						
385	230	0.02	0.005	7	14	1,600	300	100	40
386	225	"	"	"	"	12,000	4,000	800	200
387	260	"	"	"	"	10,000	Died		
388	260	"	"	"	"	1,000	800	100	40
389	250	0.01	0.0025	"	"	3,000	Died		
390	275	"	"	"	"	8,000	4,000	100	60
391	220	"	"	"	"	1,600	2,000	400	100
392	230	"	"	"	"	6,000	2,000	160	40
393	280	0.006	0.0015	"	"	1,600	1,000	60	
394	220	"	"	"	"	2,000	800	160	40
395	225	"	"	"	"	4,000	Died		
396	220	"	"	"	"	4,000	2,000	600	100
				Saline controls					
397	225	1 cc.	—	"	"	6,000	Died		
398	222	"	—	"	"	12,000	4,000	160	

II. The Effect on the Hemolysin Formation of Varying Quantities of Epinephrine, Administered during 3 Days Prior and 4 Days Subsequent to the Injection of Sheep Cells.

The first series received 0.08 mg. adrenin per kilo per day or one-fifth the amount used in the first group of experiments.   Eight rats were used, of which 6 were injected with epinephrine, 1 with saline

solution, and 1 was a normal rat. The average titer of the treated rats was 1/1800 or slightly less than normal. The depression was less marked than when the larger amounts were used.

The next three series were run in a single experiment. Fourteen rats were used. Four received daily injections of 0.02 mg. per kilo, 4, 0.01 mg. per kilo, 4, 0.006 mg. per kilo. There were 2 saline controls. The titers in all instances, when these smaller amounts were used, were normal.

DISCUSSION.

The pharmacological effects of injections of epinephrine have been the subject of extensive experiments. Very little, however, is known of its influence on the phenomena of immunity. We have noted a marked depressant effect on antibody formation of repeated injections of large quantities of epinephrine administered for several days prior and several days subsequent to the injection of antigen in normal adult albino rats. When smaller quantities were administered the same number of times, the depression was less marked. The same daily quantities administered during a shorter period depressed the titer to a less degree. The range of effective amounts of epinephrine was determined. With small quantities there is little effect or perhaps a slight rise above the normal. The quantity of epinephrine injected in the first group of experiments produced a glycosuria, which persisted during 12 hours following the injection. The rats reacted to this quantity of epinephrine by extreme restlessness followed by a short period of depression. In some instances the rats were depressed from the start and dragged their hind legs about as if they were paralyzed. This, however, wore away in a few minutes.

In a recent study on the relation of the reticulo-endothelial system to the glands of internal secretion, Leites and Riabow (13) noted a depression in the phagocytic power of the reticulo-endothelial cells following an injection of a large amount of epinephrine. This was determined by the rapidity of disappearance from the circulation of sugar of iron (ferri oxidum saccharatum) injected into the blood stream of rabbits. They contend that large amounts of epinephrine exhaust these cells. If the lymphoid and reticular elements play a

rôle in antibody formation,—and considerable evidence to this effect has already accumulated,—it is possible to explain the action of large amounts of epinephrine on antibody forming capacity in this manner.

The opposite effect noted by some observers using a single injection of a small amount of epinephrine does not in any way contradict this interpretation. We have not exhausted the possible methods of administrating epinephrine and it is possible that we shall find in some instances, when small amounts are used, a slight stimulating effect.

It has been found that removal of the suprarenals results in a depression in hemolysin forming capacity during the early period following the operation (2). The administration of large quantities of epinephrine in normal rats has a similar effect. In both instances there is apparently an exhaustion of the antibody forming mechanism.

SUMMARY.

The effect of subcutaneous injections of varying amounts of epinephrine on the hemolysin formation of normal adult albino rats was studied. In one group of experiments the rats received 0.4 mg. per kilo per day in two injections during 3 days prior and 4 days subsequent to the injection of sheep cells. Another series received the same daily amounts but only during 4 days subsequent to the injection of antigen. A third series received the same daily amounts only during 1 day prior and 2 days subsequent to the injection of sheep cells. In a second group of experiments smaller amounts of epinephrine were given during 3 days prior and 4 days subsequent to the injection of antigen. The amounts used were 1/5th, 1/20th, 1/40th, and 1/60th the daily quantity of epinephrine injected in the first group of experiments.

CONCLUSION.

Large amounts of epinephrine injected repeatedly before and after the injection of antigen depress the antibody forming capacity of normal adult albino rats to a marked degree. The depression in antibody formation is roughly proportional to the quantity of epinephrine injected. Small amounts have no detectable effect on antibody formation.

We wish to acknowledge our appreciation to Dr. David Marine for his suggestions and criticisms during the progress of this work.

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AN EXPERIMENTAL STUDY OF DIATHERMY.

VI. Conduction of High Frequency Currents through the Living Cell.

By RONALD V. CHRISTIE, M.B., Ch.B.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In this paper an attempt will be made to correlate the investigations of biophysicists with those questions which are of fundamental importance in a study of diathermy.   A survey of the literature shows that the intimate relationship of these fields has not been appreciated. Biophysicists on the one hand have paid little attention to the phenomenon of heat production with the passage of high frequency currents, while those studying diathermy have neglected the problem of cellular conduction and penetration.   It is obviously of great importance to know whether the diathermy current passes through the living cell, and if so, what are the effects produced.

The only constant effect which is known to be produced by high frequency alternating currents is that of heat production.   The chemical effects of electrolysis disappear as the alternations exceed a frequency of 5,000 to 10,000 per second (1).   The explanation for this fact lies in the rapidity of the reversal of the current which does not allow the chemical action of one phase to manifest itself before it is neutralized by the opposite phase (2).   The absence of any exciting influence on the tissues through which the current passes is probably analogous to this absence of chemical effect.   If the high frequency current is rectified so that it is not alternating in character, electrolysis and muscle excitation will be produced, even if the frequency of interruptions be as high as 500,000 per second (3).

The complexity of living tissue makes determinations extremely difficult of the actual course and means of transportation of the current through it.   Philippson (4), Fricke and Morse (5), and McClendon (6), in their work on muscle, liver, and blood cells, have shown that as the frequency of alternations is increased the impedance of the living cell decreases, meaning by impedance the sum of all the various hindrances to the passage of the current.   At a frequency of 10^5 to 10^7 cycles per second a point is reached where the impedance of living and dead cells is in close agreement.   Beyond this frequency, according to these authors, the

current passes in part at least through the cell, and not wholly through the extra-cellular fluid, as was formerly supposed. The red blood cell is surrounded by a very efficient dielectric layer, equal in thickness to a layer composed of approximately 20 to 30 carbon atoms, and allowing little or no penetration of ions. The interior of the cell has a comparatively low resistance, equivalent to a 0.17 per cent NaCl solution. The cell, therefore, acts as a condenser, which transmits the current by its capacitance. In the living body the various fascial layers also act as dielectrics (7) of varying degrees of efficiency.
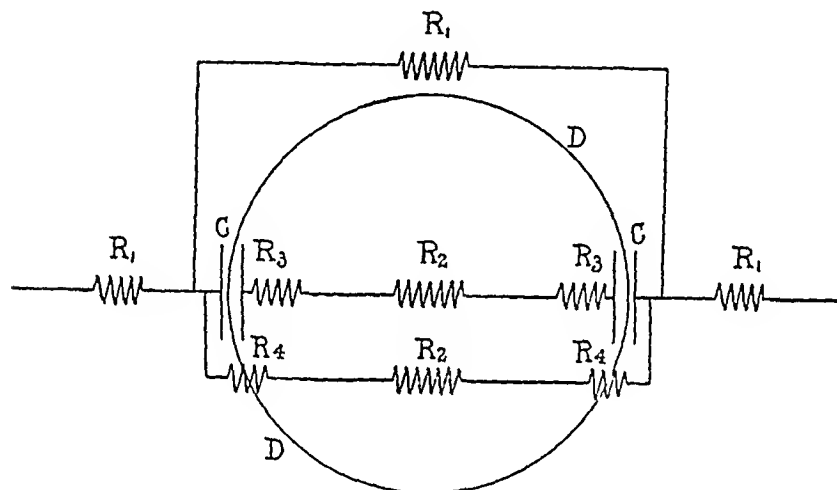
FIG. 1. Diagrammatic representation of the passage of high frequency currents through the living cell.

R_1—Resistance of extracellular fluid.
R_2—Resistance of cell interior.
R_3—Dielectric loss.
R_4—Condenser leak.
C —Capacitance of cell.
D —Dielectric layer surrounding cell.

It will be realized that the passage of high frequency currents through the living body is an extremely complex phenomenon. The whole system consists of resistances and capacitances, both in parallel and in series, some of which are stationary and some in motion. Fig. 1 represents in diagram the passage of the current through a single unit of this system—namely, the living cell. Even this representation is, of course, too simple, since the various phases in the colloidal system we call protoplasm must influence the passage of the current within

the cell (8, 9) and the protoplasm itself is constantly changing in conductivity (10).

One would expect these general principles to be applicable to the diathermy current. However, there are several differences between the currents used by the investigators mentioned and those used in diathermy which might render such an application fallacious. The diathermy current consists of pulses of impressed discontinuous oscillatory trains which probably contain many harmonics. This type of current is not necessarily comparable to a high frequency current of the pure sine wave form. Also, impedance measurements with currents of low and high voltage are not necessarily comparable (11).

There are two characteristics of the passage through living cells of low voltage high frequency alternating currents of the pure sine wave form. These, as described by the authors referred to, manifest themselves in the fact that the impedance is not changed when the cell is broken down by lysis, and in the fact that with increase of cellular concentration the impedance increment is less than that found with continuous currents.

The experiments described in this paper were planned for the purpose of finding out whether these two characteristics hold true with the currents used in diathermy.

EXPERIMENTAL.

Details of the apparatus used to produce the high frequency alternating current have been described elsewhere (12). The oscillatory current generated by the discharge of the condenser across the spark gap is of high voltage but low amperage. Owing to the high decrement of the circuit the oscillations are in the form of a series of discontinuous trains, each train being damped down before the next train commences. The frequency is 1.25×10^6 but the oscillations are not of the pure sine wave form, as harmonics are probably present.

Since it is impossible to make any accurate impedance measurements by the bridge method with this type of current, we were forced to approach the subject from a somewhat different angle from that which has been adopted by the investigators just mentioned. The experiments about to be described naturally fall into two groups, more or less independent of each other. They will, therefore, be described separately.

I. *Direct Measurement of Heat Production in Blood and Serum.*

The heat produced in blood and serum by the passage of a known quantity of current was measured by placing the blood or serum in a specially constructed glass cylinder, so arranged that temperature measurements could be made while the current was passing through it.

The material used was defibrinated sheep blood, obtained fresh from the slaughter house. The cell volume of the blood was varied by centrifugalization. Hematocrit readings were made at 2,000 revolutions per minute for 45 minutes. The cylinder was 20 cm. long and 6.5 cm. in diameter, with a capacity of 640 cc. It was closed by two cork stoppers equipped with circular lead-tin electrodes, the leads passing through the corks. Three openings on the long axis of the cylinder allowed the introduction of thermometers and the fluids to be examined. The cylinder was insulated against heat loss by wrapping it with thick felt. With this apparatus it was possible to measure the temperature changes in blood during the passage of high frequency currents through it.

To correct for heat loss during the current flow, measurements were made on the rate of cooling of water contained in the cylinder, with various differences between the temperature of the water and room temperature. When this difference amounted to 10°C. the rate of cooling was 0.04°C. per minute, showing that insulation against heat loss was fairly efficient. In the experiments to follow, however, corrections for this heat loss were made.

The strength of the current in all the experiments was 400 milliamperes, as recorded by the hot wire milliammeter and the time of exposure to the current was 10 minutes. By altering the spark gap, the milliammeter reading was kept constant throughout each experiment, so that, although the true amperage was probably changing owing to the changes of temperature in the various circuits (13), the amount of current which passed through each individual sample was approximately the same. To standardize as far as possible these changes in temperature, both the apparatus and the sample to be examined were at room temperature when each experiment began. In spite of these precautions, preliminary experiments with salt solutions of various concentrations showed that the range of error was considerable, being in the neighborhood of ± 10 per cent.

The heat production in blood and serum was measured by this method. As might be anticipated, it was found that whereas the heat production in various samples of serum was the same, that in blood increased as the cell volume was increased. For example, the ratio $\frac{H \text{ Blood}}{H \text{ Serum}}$ (where H represents heat production per unit of time) was found to increase from 1.48 to 3.13 when the cell volume was increased from 36 per cent to 72 per cent (Table I). At the same cell

volumes McClendon found the ratio $\dfrac{Z\ \text{Blood}}{Z\ \text{Serum}}$ at $F = 10^5$ to increase from 1.8 to 3.5 and $\dfrac{Z\ \text{Blood}}{Z\ \text{Serum}}$ at $F = 10^3$ to increase from 1.8 to 4.7 (Table I) (where Z represents impedance measured by the Wheatstone bridge).. It is evident from these figures that as the cell volume is increased the increase of the ratio $\dfrac{H\ \text{Blood}}{H\ \text{Serum}}$ resembles the increase in $\dfrac{Z\ \text{Blood}}{Z\ \text{Serum}}$ at $F = 10^5$ rather than at $F = 10^3$. This is especially evident at the higher cell volumes, where there is a really significant difference

TABLE I.

Impedance of Blood and Serum.

Cell vol.	H_s	H_b	$\dfrac{H_b}{H_s}$ $F = 10^3 \times 1.25$	$\dfrac{Z_b}{Z_s}$ $F = 10^5$	$\dfrac{Z_b}{Z_s}$ $F = 10^3$
per cent					
36	˙2.0	2.95	1.48	1.8	1.8
40	1.98	4.07	2.06	1.9	2.1
46.5	2.0	3.33	1.67	2.2	2.4
70	2.0	6.78	3.36	3.4	4.4
72	1.98	6.19	3.13	3.5	4.7

H = Heat produced by 400 milliamperes in 10 minutes.
Z = Impedance (data from McClendon (6)).

between $\dfrac{Z\ \text{Blood}}{Z\ \text{Serum}}$ at $F = 10^5$ and $F = 10^3$. That the ratio $\dfrac{H\ \text{Blood}}{H\ \text{Serum}}$ is as an average slightly lower than $\dfrac{Z\ \text{Blood}}{Z\ \text{Serum}}$ at $F = 10^5$ is probably of some significance, although the difference is within the limits of experimental error. Should the difference be real, it would indicate that some portion of the impedance of blood cells does not lead to heat production.

Measurements were also made on blood and blood laked with saponin but we have not published these figures, as subsequent observations showed that saponin itself produces a marked increase in conductivity.

II. *Relative Impedance and Resistance Measurements on Blood, Laked Blood, and Serum.*

A circuit was constructed whereby the relative impedance and re-sistance of two unknowns could be ascertained simultaneously. This was done to obviate the errors arising from current measurements by means of the hot wire milliammeter and from varying atmospheric conditions.

Four identical cells were constructed, each of 200 cc. capacity, and equipped with brass electrodes, the dimensions of which were 7 cm. × 4 cm. The cells were then placed in a circuit as represented in Fig. 2, so that Cell I was in series with Cell III, and Cell II in series with Cell IV. Cells I and III together were in parallel with Cells II and IV. With the same electrolyte in each of the cells, it is obvious that the amount of current, and therefore the rate of heat production in each, should be the same. To calibrate the cells, all four were filled with 0.05 M NaCl and the heat increment measured after the passage of 1,300 milliamperes for 10 minutes. This was repeated 14 times. A slight but constant discrepancy in the heat production in the respective cells was found. The average figures for the 14 observations were:
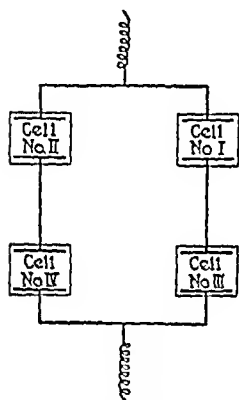
Fig. 2. Circuit used to determine the relative impedance and rate of heat production in two unknowns. Details of the apparatus are described in the text.

Cell I............................ 8.38°C.
Cell II........................... 8.69°C.
Cell III.......................... 8.33°C.
Cell IV........................... 8.57°C.

This discrepancy can only be due to some differences in the electrical constants of the cells, or differences in the calibration of the thermometers. Cell I was arbitrarily chosen as the standard and the others corrected on the basis of the above observations. The correction factors used were therefore:

1.000 for Cell   I.
0.964 for Cell   II.
1.006 for Cell III.
0.977 for Cell IV.

With these corrections the temperature increments in all 4 cells corresponded to within ± 1.7 per cent. These correction factors were used in the experiments about to be described. To correct for heat loss in the cells cooling curves were

constructed with saline and blood. The appropriate correction was applied to the observed temperature increase. All experiments were started with the contents of the cells 0.5–1.5°C. below room temperature. An effort was made to keep the environmental temperature about the cells constant.

The two unknowns, the impedances of which were to be measured, were placed in Cells I and II, respectively, while Cells III and IV were filled with 0.05 M NaCl. The current was then turned on so that the milliammeter registered 1,300, and after 10 minutes the temperature increment in all four cells was measured. (This temperature increment will be referred to as H.) Since the circuit was constructed so that, apart from the contents of the cells, its impedance was very small, we can say that the expression $\dfrac{Z_1 + Z_3}{Z_2 + Z_4}$ represents the inverse ratio of the currents passing through the two parallel arms of the circuit (where Z_1 represents the impedance of the contents of Cell I, Z_2 the impedance of the contents of Cell II, etc.). The amount of current passing through Cell I is the same as that passing through Cell III, and the amount of current passing through Cell II is the same as that passing through Cell IV. Hence we can write:

$$\frac{Z_1 + Z_3}{Z_2 + Z_4} = \frac{I_2}{I_1} = \frac{I_4}{I_3}$$

Since Cells III and IV contain an electrolyte of the same conductivity, we can write $\dfrac{I_4}{I_3} = \dfrac{\sqrt{H_4}}{\sqrt{H_3}}$.

It follows that:

$$\frac{Z_1 + Z_3}{Z_2 + Z_4} = \frac{\sqrt{H_4}}{\sqrt{H_3}} \dotfill (1)$$

Since Z_3 and Z_4 are identical, the figure $\dfrac{\sqrt{H_4}}{\sqrt{H_3}}$ will tell us whether $\dfrac{Z_1}{Z_2}$ is greater or less than unity.

If we assume that the contents of the four cells have a similar dielectric constant, an assumption which seems reasonable since they consist of electrolytes with no great divergence of conductivity, we can obtain from these heat measurements a figure which will represent quantitatively the ratio $\dfrac{R_1}{R_2}$, where R represents that part of the impedance which leads to heat production. Since the amount of current flowing through Cells I and III is the same and that flowing through II and IV is the same, we can write $\dfrac{R_1}{R_3} = \dfrac{H_1}{H_3}$ and $\dfrac{R_2}{R_4} = \dfrac{H_2}{H_4}$. It follows that

$\dfrac{R_1}{R_3}\Big/\dfrac{R_2}{R_4} = \dfrac{H_1}{H_3}\Big/\dfrac{H_2}{H_4}$, which can be written $\dfrac{R_1}{R_3}\times\dfrac{R_4}{R_2} = \dfrac{H_1}{H_3}\times\dfrac{H_4}{H_2}$. Since Cells III and IV both contain 0.05 M NaCl, R_4 and R_3 can be cancelled out so that:

$$\frac{R_1}{R_2} = \frac{H_1}{H_3}\times\frac{H_4}{H_2}\dots\dots\dots\dots\dots\dots\dots\dots\dots(2)$$

The accuracy of this method was checked by a series of observations on various electrolyte solutions, of which the conductivity had been measured by a Wheatstone bridge at a frequency of 10^3. There is no reason to suppose that conductivity measurements by the low frequency Wheatstone bridge should not be applic-

TABLE II.

Saline and Saponin Controls.

Contents of cells			$\dfrac{R_1}{R_2}$		$\dfrac{Z_1+Z_3}{Z_2+Z_4}$	
I	II	III and IV	$F = 10^5$ $\times 1.25$	$F = 10^3$	$F = 10^5$ $\times 1.25$	$F = 10^3$
0.04 M NaCl	0.06 M NaCl	0.05 M NaCl	1.474	1.439	1.171	1.195
			1.429	1.439	1.198	1.195
			1.427	1.439	1.201	1.195
			1.473	1.439	1.211	1.195
0.55 M NaCl	0.05 M NaCl	0.05 M NaCl	0.915	0.916	0.957	0.955
			0.930	0.916	0.971	0.955
0.05 M NaCl	0.05 M NaCl + 1 per cent saponin	0.05 M NaCl	1.317	1.302	1.130	1.132
			1.278	1.302	1.139	1.132

Z = Impedance of contents of cell.
R = Impedance represented by heat production.

able to the diathermy current when simple electrolytes are used. In all the experiments the unknowns were placed in Cells I and II while Cells III and IV were filled with 0.05 M NaCl. With 0.04 M NaCl and 0.06 M NaCl as the unknowns, the average values for $\dfrac{R_1}{R_2}$ and $\dfrac{Z_1 + Z_3}{Z_2 + Z_4}$ were 1.451 and 1.195 with the diathermy current, 1.439 and 1.195 with the Wheatstone bridge. With 0.055 M NaCl and 0.050 M NaCl the figures were 0.922 and 0.964 with diathermy and 0.916 and 0.955 with the Wheatstone bridge. With 0.05 M NaCl and 0.05 M NaCl + 1 per cent saponin, the figures were 1.297 and 1.134 with diathermy and 1.302 and 1.132 with the Wheatstone bridge (Table II). These controls clearly show that even slight differences in impedances can be measured by this method with a considerable degree of accuracy.

Similar experiments were carried out with blood cells and laked blood cells, and also with blood cells and serum, as the unknowns. The material used was defibrinated ox blood, obtained fresh from the slaughter house.   The serum was separated and the cell volume varied by centrifugalization.   The blood cells were laked by freezing and thawing.   As in the control experiments, the unknowns were placed in Cells I and II, while Cells III and IV were filled with 0.05 M NaCl. It was found that whereas laking produced no appreciable change in

TABLE III.

Impedance of Blood and Laked Blood.

Hematocrit reading	$F = 10^4 \times 1.25$ $\dfrac{R \text{ Blood}}{R \text{ Laked blood}}$	$F = 10^2$ $\dfrac{Z \text{ Blood}}{Z \text{ Laked blood}}$	$F = 10^4 \times 1.25$ $\dfrac{Z \text{ Blood} - Z\ 0.05 \text{ M NaCl}}{Z \text{ Laked blood} - Z\ 0.05 \text{ M NaCl}}$
73	0.976	1.31	0.986
	0.985	1.31	0.990
50	0.925		0.959
	0.945		0.969
47	0.987	1.092	1.013
	0.997		1.006
	0.990		1.013
39	0.965		0.969
	0.990		0.996
	0.963		0.975
Average	0.973		0.997

Z = Impedance.
R = Impedance represented by heat production.

the impedance of blood, the rate of heat production was increased by about 3 per cent.   The average of the 10 observations made was 0.997 for the value of $\dfrac{Z \text{ Cells} + Z\ 0.05 \text{ M NaCl}}{Z \text{ Cells laked} + Z\ 0.05 \text{ M NaCl}}$ and 0.973 for the value of $\dfrac{R \text{ Cells}}{R \text{ Cells laked}}$ (Table III).   Since presumably all the impedance of laked blood is ohmic in character, this can only mean that about 3 per cent of the impedance of intact cells is inductive, and therefore does not lead to the production of heat.

With blood of 72 per cent cell volume and serum as the unknowns, the ratio $\dfrac{R\,\text{Blood}}{R\,\text{Serum}}$ was found to average 2.5. McClendon found that at the same cell volume $\dfrac{Z\,\text{Blood}}{Z\,\text{Serum}}$ was 3.5 at $F = 10^6$ and 4.7 at $F = 10^3$. Thus we again have $\dfrac{R\,\text{Blood}}{R\,\text{Serum}}$ somewhat lower than $\dfrac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at the same frequency and very much lower than $\dfrac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at low frequencies (Table IV). The discrepancy between $\dfrac{R\,\text{Blood}}{R\,\text{Serum}}$ and $\dfrac{Z\,\text{Blood}}{Z\,\text{Serum}}$ at

TABLE IV.

Impedance of Blood and Serum.

Cell vol.	$\dfrac{R\,\text{Blood}}{R\,\text{Serum}}$	$\dfrac{Z\,\text{Blood}}{Z\,\text{Serum}}$	$\dfrac{Z\,\text{Blood}^*}{Z\,\text{Serum}}$	$\dfrac{Z\,\text{Blood}^*}{Z\,\text{Serum}}$
	$F = 10^6 \times 1.25$	$F = 10^3$	$F = 10^3$	$F = 10^3$
per cent				
72	2.60	4.36	3.5	4.7
72	2.42	4.41	3.5	4.7

Z = Impedance.
R = Impedance represented by heat production.
* Data from McClendon (6).

the same frequency confirms the previous observation that part of the impedance of blood cells does not lead to the production of heat. The difference, however, is more than one would expect from the results obtained on whole and laked blood. Some of this difference can no doubt be explained by differences in the technic employed for making hematocrit readings by McClendon and ourselves. However, if we make use of the impedance measurements at $F = 10^3$ (Table IV), as a standard of comparison, a considerable discrepancy still exists $\left(\dfrac{R\,\text{Blood}}{R\,\text{Serum}} = 2.5 \text{ and } \dfrac{Z\,\text{Blood}}{Z\,\text{Serum}} = 3.3\right)$.

DISCUSSION.

The results of these experiments seem to show pretty conclusively that, with regard to its passage through biological media, the dia-

thermy current behaves in the same manner as low voltage high frequency currents of the pure sine wave form. With both, the laking of cells produces no change in impedance, and with both, an increase in cellular concentration of blood produces an impedance change which is characteristically lower than that found with low frequency currents. The passage of the diathermy current through the living cell can then be represented by Fig. 1. The cell membrane is a very efficient condenser which transmits the current by its capacitance with little or no dielectric loss and consequently little or no stress and strain on the "cell wall." Cellular massage is a term commonly met with in the literature on diathermy. There is no evidence to show that either this, or the electromechanical vibration which is said to occur with currents of a higher frequency, exists.

From theoretical considerations we should not expect that the addition of a glucoside such as saponin to an electrolyte would produce any appreciable change in the conductivity. To our surprise, however, we found that a very definite increase in conductivity occurred (Table II). This increase appears to be independent of voltage and frequency, so it must be a true conductivity change. No attempts were made to determine the purity of the saponin used, but it was obtained from C. A. F. Kahlbaum of Berlin and classified as purified saponin. Others working on the conductivity of biological media under various conditions seem to be unaware of this phenomenon (6) and we, ourselves, were for some time led astray by it.

SUMMARY.

1. A method is described for measuring the relative impedance of living cells to diathermy currents.

2. The diathermy current penetrates the living cell, and heat production is intracellular as well as extracellular.

3. A small proportion of the impedance of living cells to the diathermy current seems not to lead to the production of heat.

4. Evidence is given that the addition of saponin produces an appreciable increase in the conductivity of an electrolyte. Its use is therefore contraindicated when electrical measurements are being made on biological material.

5. The currents used in diathermy behave as do high frequency

currents of the pure sine wave form in respect to their passage through biological material.

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STUDIES ON BACILLUS TYPHOSUS TOXIC SUBSTANCES.

I. Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.*

By GREGORY SHWARTZMAN, M.D.

(From the Laboratories of the Mount Sinai Hospital, New York.)

PLATES 6 AND 7.

INTRODUCTION.

Brieger (1) initiated the studies on toxic substances derived from *B. typhosus* cultures. Schütze (2) claimed that these substances possessed no antigenic properties. Further work on the nature of the toxic factors was done by Sirotinin (3), and Beumer and Peiper (4). Bitter (5), Chantamesse (6), Besredka (7), Rodet and Lagriffoul (8), Kraus and Stenitzer (9), Arima (10) and others obtained immune sera which were supposed to neutralize the toxic substances derived from *B. typhosus* cultures grown in fluid and solid media. Pfeiffer and Bessau (11) showed that immune sera had no superior neutralizing properties over normal sera and that there was no neutralization according to the law of multiple proportions. The toxic substances of *B. typhosus* have been claimed to be heat resistant by Sirotinin (3), Chantamesse (6) and Besredka (7). According to Kraus and Stenitzer (9) these substances are not influenced by light or by exposure to room temperature. The majority of the authors considered the toxic substances as endotoxins (Pfeiffer (11), Besredka (7) and others). Kraus and Stenitzer (9) and later Arima (10) were of the opinion that the typhoid cultures contain both endotoxins and exotoxins. The symptoms produced by the toxic substances are devoid of any specific features. In acutely poisoned rabbits they consist of convulsions, Cheyne-Stokes respiration, diarrhea, paralysis and, possibly, swelling of Peyer's patches (Arima (10)). Similar symptoms have been demonstrated by injection of anaphylatoxins (Friedberger (12)).

On the basis of the work thus far mentioned, a general belief arose that no true toxins exist in *B. typhosus* cultures. In fact, the classical requirements for demonstration of a true toxin, namely, heat lability, serum neutralization in multiple proportions and specific pathological effect upon animals were not fulfilled. The subject has been revived by Zinsser (13) in recent years. The toxic substances obtained from fluid cultures of various microorganisms, including *B. typhosus*,

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were called by him x-substances. They produced no specific pathological changes when injected into animals. Antigenic properties could not be determined because of fluctuations in the response of the animals and inability to continue immunization over a prolonged period of time. The x-substances were also heat resistant, were more toxic for rabbits than for guinea pigs and their effect upon animals was always accompanied by a short but definite incubation period. Zinsser thought that they could not be dismissed merely as split products and considered them somewhat similar to exotoxins as reported by Kraus and Stenitzer, and by Arima.

The present author has carried out experiments as preliminary to the work here to be reported, which can be summarized as follows:

Whole cultures and filtrates of cultures of *B. typhosus* grown under various conditions presumably favorable to the production of powerful toxins were injected intravenously into rabbits. The symptoms produced by the intravenous injections were similar to those described by previous authors. The susceptible animals became sick shortly after the injection. Before death there was very profuse diarrhea, increased respiration, paralysis and, frequently involuntary muscular contractions. Analysis of the highly inconstant results of titration of the toxic effect in a large group of rabbits showed the impossibility of establishing any relation between the dose of the toxic substances injected and the reaction of the animals. The inconstancy of the effect was most likely due to individual variations in the susceptibility of the animals to the effect of these substances.

The effect of *B. typhosus* culture filtrate upon the skin of normal rabbits was also studied. About 50 per cent of all the normal rabbits tested by skin injections of the filtrate showed no reactions whatsoever. In the remaining rabbits the skin injections produced erythemas. Only a small percentage of the positively reacting animals (12 per cent) gave well pronounced erythemas. A majority of positive rabbits reacted weakly. Moreover, different areas of the skin of the abdomen of the same rabbits presented considerable variations in the intensity of reactions to *B. typhosus* culture filtrate.

As can be seen from this summary, the general response and the skin reactions of rabbits to *B. typhosus* culture filtrate could not be used as criteria for studies on the nature of the toxic factors of these filtrates. Further experiments now to be described demonstrated a phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

skin were made.   Rabbit 14-2 showed no reaction in the course of 24 hours following the intravenous injection.

Protocol 2.—4 areas of the skin of the abdomen of Rabbit 7-6 were injected each with 0.25 cc. of T. D. T_{240} filtrate.   20 hours later there were "2 +" erythemas at the site of skin injections.   24 hours after the skin injections this rabbit was injected intravenously with the filtrate in a dose of 1 cc. per kilo of body weight.   The previously injected skin areas showed discoloration in about 2 hours after the intravenous injection.   The reactions again, progressively, increased and 4½ hours after the intravenous injection they were extremely severe.   The areas were hemorrhagic.   They appeared dark blue, glossy and swollen.   The upper right corner reaction measured 3½ × 3 cm., the lower right 2 × 2½ cm., the upper left 2 × 2 cm. and the lower left 1½ × 3 cm.   Rabbit 7-6 was reexamined 24 hours later.   The areas were then black with a dark red zone at the periphery.   The size remained unchanged.   It appeared that the reaction reached its maximum size in about 5 hours after the intravenous injection.   The healing of the hemorrhagic areas was slow.   Sloughs formed in about 48 hours after the intravenous injection.   The sloughs were followed by scab formation, their gradual separation and scarring.   The complete process of healing took about 8 days.

Histological examinations of the hemorrhagic areas were made.

Sections of these areas from Rabbit 14-1 obtained 5 hours after the intravenous injection can be described briefly, as follows:

The general impression was that of the severest type of hemorrhage and necrosis.   The skin was edematous in places.   Some of the blood vessels were ruptured.   The subcutaneous tissue was engorged with blood.   There was also an extensive migration of polymorphonuclear neutrophil leucocytes.   There was observed pronounced necrobiosis of these cells located inside and outside the blood vessels.   Some of the blood vessels contained small parietal thrombi.   While it was clear that the process affected the veins, it remained unsettled whether there was any primary injury to the arteries.   Some of the arteries were found normal.   Others were almost entirely obliterated.   The obliteration, however, was probably secondary to the hemorrhagic infiltration outside the arteries.   Occasionally, hyalinization was seen in the blood vessels.   The hemorrhage and necrosis extended to the corium of the skin, which was thin and broken in places.

The sections of the hemorrhagic areas of Rabbit 7-6 made 24 hours after the intravenous injection were almost identical with the sections of Rabbit 14-1.

More extensive histological studies of the phenomenon are under progress.

As is seen from these protocols, a phenomenon of local skin reactivity to *B. typhosus* culture filtrate was observed.   The reactivity was due to skin injection of the filtrate 24 hours prior to the intravenous injection of the same filtrate.   The local response was that of severest hemorrhage and necrosis and was fully developed 4 to 5 hours

after the intravenous injection.[1] This phenomenon was reproduced in many rabbits.

In order to determine the characteristic features of the phenomenon the following points were investigated:

1. Susceptibility of Normal Rabbits to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrates.

In the course of the work it was observed that certain animals did not respond to the above described treatment. Statistical data were accumulated in order to determine the percentage of normal rabbits which were susceptible to the phenomenon of local reactivity to *B. typhosus* culture filtrates.

In this group of experiments filtrates of Strain T_L or T_{240} cultures in tryptic digest broth were employed. Each area of the skin was injected with 0.25 cc. of undiluted or diluted 1:2 culture filtrate. The dose for intravenous injection varied from 1 to 3 cc. per kilo of body weight. The interval between the skin and intravenous injections was from 20 to 24 hours. Some of the animals died shortly after the intravenous injection (1 to 3 hours) or in the course of the following 48 hours. A considerable percentage of rabbits survived 48 hours. Although in many cases it was already possible to make readings of the reactions 2 hours after the intravenous injection the rabbits which died earlier than 3 hours after the intravenous injection were not taken into consideration in this part of the work.

In the animals which were considered resistant to the phenomenon there was no local hemorrhagic necrosis following the intravenous injection. When, prior to the intravenous injection, there was erythema at the site of skin injections, it became more pronounced 4 to 5 hours after the intravenous injection. Frequently swellings appeared. 24 hours later the skin appeared normal again.

The positively reacting animals showed very severe hemorrhagic necrosis at the site of preliminary skin injections. No mild reactions which would constitute an intermediate group between the negative and positive animals were obtained under the conditions of this part of the work. Of the 212 animals tested in this manner, there were 45 negatively reacting rabbits and 167 rabbits which showed severe reactions (approximately, 78 to 79 per cent positive animals).

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[1] The factors which induced the local skin reactivity are termed *"skin preparatory factors"* and those involved in production of local hemorrhagic reactions following the intravenous injection *"reacting factors."*

TABLE I.

The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.

No. of rabbits	Dose of each skin injection	Dose of intravenous injection per kilo of body weight	Filtrates used for skin and intravenous injections	Size, intensity and type of reaction in various areas of the skin of the abdomen			
	cc.	cc.		The upper right area of the abdomen	The lower right area of the abdomen	The upper left area of the abdomen	The lower left area of the abdomen
I	0.5	2.8	T.D.T$_L$	Hem. 4+ 2 × 2½ cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 3 × 2 cm.
II	"	2	T.D.T$_{240}$	–	–	–	–
III	"	3	T.D.T$_L$	Hem. 4+ 2 × 3 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 4 × 2 cm.	Hem. 4+ 3 × 2½ cm.
IV	"	"	"	–	–	–	–
V	0.25	0.5	"	Hem. 4+ 2 × 1½ cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 3 × 1 cm.
VI*	"	"	"	–	–	–	–
VII	"	3	"	Hem. 4+ 2 × 1 cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 3 × 2 cm.	Hem. 4+ 2 × 2 cm.
VIII	"	0.2	T.D.T$_{240}$	Hem. 4+ 2 × 2 cm.	Hem. 4+ 3 × 1 cm.	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1½ × 1½ cm.
IX	"	3	T.D.T$_L$	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1 × 1 cm.	Hem. 4+ 2 × 3 cm.	Hem. 4+ 3 × 1 cm.
X	"	"	"	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1 × 2 cm.	Hem. 4+ 3 × 1 cm.	Hem. 4+ 3 × 1 cm.
XI	"	"	"	Hem. 4+ 1 × 2 cm.	Hem. 4+ 1 × 1 cm.	Hem. 4+ 4 × 4 cm.	Hem. 4+ 2½ × 2 cm.
XII**	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+ 2 × 2 cm.	Hem. 4+ 1½ × 2 cm.
XIII	"	"	"	Hem. 4+ 1½ × 1½ cm.	Hem. 4+ 1½ × 2 cm.	Hem. 4+ 2½ × ½ cm.	Hem. 4+ 1½ × 2 cm.

			Reaction 1	Reaction 2	Reaction 3	Reaction 4
XIV	0.5	0.5	"	−	−	−	−
XV	0.8	"	"	Hem. 4+ / 3½ × 3 cm. / Hem. 4+	Hem. 4+ / 2 × 2 cm. / Hem. 4+	Hem. 4+ / 2 × 2 cm. / Hem. 4+	Hem. 4+ / 1½ × 3 cm. / Hem. 4+
XVI***	2	0.25	"	−	−	−	−
XVII*	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XVIII***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XIX	"	"	"	1 × 1 cm.	2 × 1 cm.	1½ × 2 cm.	1½ × 1½ cm.
XX***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXI***	"	"	"	Hem. 4+	Hem. 4+	Hem. 4+	Hem. 4+
XXII***	"	"	"	....	....	....	....
XXIII***	"	"	"	....	....	....	....
XXIV****	"	"	"	....	....	....	....

........, not protocolled.

−, no reaction.

Hem, hemorrhagic.

4+, very severe.

* Died in ½ hour after intravenous injection. No reaction was seen yet.

** Confluent reactions.

*** Not measured. Approximately 2 × 2 cm. in size each.

**** Died in 1 to 2 hours after intravenous injection. Beginning of reaction.

2. The Uniformity of the Hemorrhagic Reaction in Various Areas of the Skin of the Abdomen.

Experiments were carried out in order to determine whether there was a uniform response of different areas of the skin of the abdomen to the intravenous injection of *B. typhosus* culture filtrates.

Protocol 1.—Rabbit 43-4 received injections of T. D. T_L filtrate into 5 areas, namely, upper and lower right and left corners and the center of the skin of the abdomen. 0.1 cc. was injected into each area. The next day there was a slight erythema (1+) in each injected area. 24 hours after the skin injections the rabbit received 3 cc. per kilo of body weight of the same filtrate intravenously. 4 hours later the previously injected skin areas were severely hemorrhagic. There was no appreciable difference in the *severity* of the reaction in different areas. The upper right area measured $1\frac{1}{2} \times 1$ cm., the other areas were 1×1 cm. in size. 24 hours following the intravenous injection no change in the size and type of reaction was observed.

As is seen from this experiment, 5 different areas of the skin of the abdomen responded to intravenous injection of the filtrate with an equal degree of severity and showed no appreciable difference in size of the reactions.

In view of the importance attached to these findings it was deemed necessary to extend the work to a large group of animals, in which the doses for both skin and intravenous injections varied to a certain extent.

Protocol 2.—A group of 24 rabbits was used. The conditions of the experiments and the results are summarized in Table I. It will be noted from this table that the positively reacting animals showed uniformly extremely severe hemorrhagic reactions in prepared skin areas following intravenous injections of *B. typhosus* culture filtrates in various doses. The only variations observed were in the size of the reaction. The smallest reaction was 1×1 cm. and the largest 4×4 cm. The size of the reaction did not definitely depend on the amount of filtrate used for the skin preparation. Injection of 0.25 cc. into the skin, frequently led to reactions as large and larger than the injection of 0.5 cc. of the filtrates. For the same animals variations in the size of different areas lay between 1 and 3 cm. in diameter, as is seen from Animal X, in which the widest variations were obtained. Ordinarily, the difference was 1 to 2 cm. Occasionally confluent reactions were obtained. No well marked relationship between the intravenous dose and the size of the reaction of the skin was observed under the conditions of these experiments.

3. Titration of the Skin Preparatory Factors of B. typhosus Culture Filtrates.

Protocol 1.—In order to titrate the skin preparatory factors of *B. typhosus* culture filtrates various dilutions were injected into the skin of the abdomen. 24 hours later the undiluted filtrate was injected intravenously in a dose of 3 cc. per kilo of weight. Readings of the reactions were made 5 and 24 hours after the intravenous injections. In this group of experiments T. D. T_L filtrate was employed.

The results obtained on 12 rabbits susceptible to the phenomenon can be summarized as follows:

The filtrate diluted up to 1:4 was consistently able to induce the local skin reactivity. Reactions following the intravenous injections were severely hemorrhagic, necrotic and varied in size from 1 × 1 cm. to 3 × 2 cm.

There were observed fluctuations in the preparatory effect of higher dilutions. Filtrate dilutions from 1:8 to 1:64 were able to induce local skin reactivity in some animals. The severity of the reactions following the intravenous injections varied from slight blue (1+) to deep blue with an angry red zone at the periphery (4+). It would be of interest to determine whether the fluctuations observed would serve as an indicator of the degree of susceptibility of the rabbits to the phenomenon of local skin reactivity to *B. typhosus* culture filtrates.

Dilutions higher than 1:64 failed to induce the local skin reactivity to *B. typhosus* culture filtrate.

Similar results were obtained with a mixture of equal parts of filtrates derived from different strains (Mt. Sinai, 240, 215_A, 215_B, T_L).

4. Relation of Intensity of Erythema Following Preparatory Skin Injection to the Local Hemorrhagic Reaction Produced by Intravenous Injection of B. typhosus Filtrate.

The purpose of the observations reported here was to determine whether the skin reaction following the preparatory skin injection had any influence on the size and severity of the local hemorrhagic response produced by intravenous injection of *B. typhosus* culture filtrate.

Rabbits were injected into 4 areas of the skin of the abdomen with 0.25 cc. of T. D. T_L filtrate. The intensity of the erythema obtained 20 to 24 hours later

TABLE II.

The Relation of the Erythema Following Skin Injection of B. typhosus Filtrate to the Hemorrhagic Reaction Following Intravenous Injection of the Same Filtrate.

No. of rabbits	Upper right corner		Lower right corner		Upper left corner		Lower left corner	
	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection	Intensity of erythema after skin injection	Size and intensity of reaction after intravenous injection
XXVI	Negative	3.5 × 4.5 cm. Hem. 4+	Negative	5 × 3 cm. Hem. 4+	—	—	—	—
XXVII	2+	2 × 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	2+	3 × 1 cm. Hem. 4+	2+	1½ × 1 cm. Hem. 4+
XXVIII	Doubtful	1½ × 1 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+	Doubtful	3 × 2 cm. Hem. 4+	Doubtful	2 × 2 cm. Hem. 4+
XXIX	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1½ cm. Hem. 4+	3+	1 × 1 cm. Hem. 4+	—	—
XXX	3+	1 × 1½ cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	1+	1 × 1½ cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+
XXXI	2+	2 × 3½ cm. Hem. 4+	2+	4 × 1½ cm. Hem. 4+	2+	4 × 3 cm. Hem. 4+	"	1½ × 2 cm. Hem. 4+
XXXII	Negative	2 × 2 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	Negative	2½ × 1½ cm. Hem. 4+	—	—
XXXIII	"	1½ × 1 cm. Hem. 4+	—	—	1+	1 × 1 cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+
XXXIV	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	—	—	"	1½ × 2 cm. Hem. 4+
XXXV	2+	1 × 2 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+	4+	1½ × 1 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+

Case			Doubtful		Doubtful		Negative	
XXXVI	3+	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	2+	1½ × 1 cm. Hem. 4+	Negative	1½ × 2 cm. Hem. 4+
XXXVII	1+	1½ × 1 cm. Hem. 4+	Negative	1 × 1 cm. Hem. 4+	2+	1 × 2 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XXXVIII	Negative	3 × 3 cm. Hem. 4+	"	3 × 3 cm. Hem. 4+	Negative	2 × 2 cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XXXIX	2+	2½ × 2 cm. Hem. 4+	"	3 × 1½ cm. Hem. 4+	2+	1 × 3½ cm. Hem. 4+	"	1 × 1 cm. Hem. 4+
XL	½+	1 × 2 cm. Hem. 4+	—	—	2+	1 × 1 cm. Hem. 4+	"	1 × 1½ cm. Hem. 4+
XLI	Negative	2½ × 2 cm. Hem. 4+	—	—	Negative	2 × 2 cm. Hem. 4+	—	2 × 2 cm. Hem. 4+
XLII	"	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1½ cm. Hem. 4+	3+	1½ × 2 cm. Hem. 4+	—	2 × 1 cm. Hem. 4+
XLIII	"	1 × ¾ cm. Hem. 4+	2+	1 × 1 cm. Hem. 4+	—	—	Negative	—
XLIV	3+	1½ × 1½ cm. Hem. 4+	2+	1½ × 1½ cm. Hem. 4+	Negative	1½ × 1 cm. Hem. 4+	1+	—
XLV	Negative	3 × 2½ cm. Hem. 4+	Negative	2 × 1 cm. Hem. 4+	—	—	Negative	2½ × 2½ cm. Hem. 4+
XLVI	½+	1½ × ½ cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+	1+	1 × 1 cm. Hem. 4+
XLVII	2+	1 × 1 cm. Hem. 4+	½+	1 × 1 cm. Hem. 4+	—	—	—	—
XLVIII	4+	2 × 2 cm. Hem. 4+	2+	2 × 2 cm. Hem. 4+	4+	2 × 2 cm. Hem. 4+	—	—

—, not tested.

was recorded.   24 hours after the preparatory injections the same filtrate was injected intravenously into these animals.   The dose varied from 2 to 3 cc. per kilo of body weight.   Readings recorded here were made 24 hours after the intravenous injections.

Some of the animals died 1 to 2 hours after the intravenous injection.   The readings of these animals were not included in the protocols.

In Table II, animals susceptible to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate are recorded.   It is seen from this table that a considerable number of areas which did not react to the local injection responded severely locally to the intravenous injection.   In addition, areas which had erythemas of various intensity showed an approximately equally active response to the intravenous injections.

In this experiment there were also 5 rabbits which were not included in Table II.   2 animals showed no erythema following the preparatory skin injections.   3 rabbits had erythema of various intensity from 1+ to 4+.   All these animals were entirely resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate.

As is seen from the observations reported above, there was no relationship between the intensity of the erythema following the preparatory skin injections and the size and intensity of the local hemorrhagic response to intravenous injections of *B. typhosus* culture filtrate.   Evidently, the local trauma produced by the preparatory skin injections was not responsible for the skin localization of the toxic factors introduced by the intravenous route.   This finding was confirmed by the observation that some animals resistant to the phenomenon of local hemorrhagic response showed erythemas of various intensity after the preparatory skin injections.

5. Attempts to Produce Local Skin Reactivity to B. typhosus Culture Filtrate by Skin Injections of Various Substances.

A series of experiments was performed in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate could be produced by skin injections of various substances.

A: The purpose of the following experiment was to determine whether uninoculated culture medium by itself was able to induce the local skin reactivity to *B. typhosus* culture filtrates.

Protocol 1.—5 rabbits were used for this experiment. The upper and lower right and upper left areas of the skin of the abdomen were injected each with 0.5 cc. of sterile tryptic digest broth. The lower left areas were injected with 0.5 cc. of *B. typhosus* culture filtrate. 24 hours later these rabbits were injected intravenously each with *B. typhosus* culture filtrate. The dose was 3 cc. per kilo of weight. 1½ hours after the intravenous injections 1 rabbit died. No readings were made. 5 hours after the intravenous injections the remaining rabbits showed no reactions in the upper and lower right and upper left areas. The lower left areas of the skin of these rabbits showed very severe hemorrhagic necrosis. The size of the reactions varied from 2½ × 2½ cm. to 3½ × 4 cm.

As is seen from this experiment, sterile tryptic digest broth failed to produce local skin reactivity to *B. typhosus* culture filtrate.

B: Experiments were performed in order to determine whether culture filtrates of various strains of streptococci were able to induce local skin reactivity to *B. typhosus* culture filtrate:

Protocol 1.—In addition to the *B. typhosus* culture filtrate, 2 strains of green producing streptococci (530 and 941) were employed in this experiment. Both strains of streptococcus were isolated from the blood of cases of subacute bacterial endocarditis. The culture filtrates were made in a manner identical with that employed for the preparation of *B. typhosus* culture filtrates.

Rabbits 14-3 and 14-4 received into the upper and lower right areas of the skin injections of streptococcus filtrates 530 and 941, respectively. The upper and lower left areas were injected with T. D. T_L filtrate. 24 hours after the skin injections no reactions were seen. The rabbits were immediately injected intravenously with T. D. T_L filtrate in a dose of 3 cc. per kilo of weight. 5 hours after the intravenous injections no reactions were seen in the upper and lower right areas. The upper and lower left areas, however, showed very severe hemorrhagic reactions. The size of the reactions varied from 1 × 1 cm. to 3 × 2 cm.

Protocol 2.—For this experiment filtrates of 2 strains of *pyogenes Streptococcus hæmolyticus* were prepared in the same manner as *B. typhosus* culture filtrates. In addition toxin of *Streptococcus erysipelatis*, kindly sent to me by Dr. K. Birkhaug under the name E_1—E_3, was employed. A group of 10 rabbits was prepared by skin injections of these filtrates into the upper and lower right and upper left areas. The lower left areas were injected with T. D. T_L filtrate. 24 hours later the rabbits were each injected intravenously with T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. In 1 to 2 hours after the intravenous injections 3 rabbits died. Readings were made 5 hours after the intravenous injections. In 6 rabbits the lower left areas showed very severe hemorrhagic reactions (4+) which varied in size from 1 × 1 cm. to 3 × 2 cm. The other prepared areas were entirely negative. 1 rabbit reacted negatively in all the 4 prepared areas.

It is evident from the experiments of Protocols 1 and 2 that no local skin reactivity to *B. typhosus* culture filtrate was induced by skin injections of 5 different streptococcus culture filtrates. The susceptibility of the rabbits employed to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate was controlled.

C: In this group of experiments the skin of rabbits was prepared by injections of turpentine and 24 hours later *B. typhosus* culture filtrate was injected intravenously.

Protocol 1.—Rabbits 49-4, 49-5, 49-6 were injected with turpentine into 4 areas of the skin of the abdomen. The upper and lower right areas were injected with dilution 1:10 and the upper and lower left areas with dilution 1:5. 24 hours later there were no abscesses in turpentine injected areas, but they were distinctly red. The intravenous injections of potent *B. typhosus* culture filtrate (T. D. T_L) in a dose of 3 cc. per kilo of weight produced no reactions in the prepared areas.

Protocol 2.—The upper and lower right and left areas of the skin of the abdomen of Rabbit 6-3 and 6-9 were injected with 0.2 cc. of undiluted turpentine and turpentine diluted 1:10, 1:20 and 1:40, respectively. 24 hours later the upper right corners of the skin of the abdomen of both animals showed well formed abscesses, the remaining areas showed reddening, but no pus was seen. 24 hours after the skin injections, T. D. T_{240} filtrate, which proved to be potent on the day of the experiment, was injected intravenously into these animals. The dose was 3 cc. per kilo of weight. No local skin reactions followed the intravenous injections.

From the observations reported in this part of the work it was concluded that non-specific irritating substances such as sterile tryptic digest broth and turpentine, and culture filtrates of certain microorganisms biologically unrelated to *B. typhosus* could not substitute the skin preparatory factors of *B. typhosus* culture filtrate. Additional studies are under way in order to determine whether the local skin reactivity to *B. typhosus* culture filtrate can be produced by microorganisms biologically related to *B. typhosus*.

6. The Effect of Heat upon the B. typhosus Skin Preparatory Factors.

To determine the effect of heat upon the skin preparatory factors a number of experiments was made:

Protocol 1.—Rabbits 28-0, 28-1 and 28-2 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated T. D. T_L diluted 1:2, with the same dilutions of T. D. T_L heated to 60°C. for 1 hour with 1:2 T. D. T_L heated in the Arnold steam sterilizer for 1 hour and with 1:2 T. D.

T_L heated in the autoclave for 45 minutes. 24 hours after the skin injections Rabbits 28-0 and 28-1 showed no reactions and Rabbit 28-2 had a diffuse redness extending over the entire skin of the abdomen (2+). 24 hours after the skin injections these rabbits received intravenously unheated T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 5 hours after the intravenous injections the upper and lower right and upper left areas of all 3 rabbits showed severe hemorrhagic reactions which varied in size. The lower left areas were entirely negative. Rabbit 28-2 died 6 hours after the intravenous injection. Rabbits 28-0 and 28-1 showed no change in reactions 24 hours after the intravenous injection.

These experiments were repeated on 10 more rabbits with identical results. The slight variations in the size of severe hemorrhagic reactions could be disregarded.

As is seen from these observations, the skin preparatory factors of this strain of *B. typhosus* culture filtrate could be inactivated by heating in the autoclave for 45 minutes. Lower temperatures had no appreciable effect on the ability of the filtrate to induce the local skin reactivity.

Protocol 2.—Similar experiments were performed with a filtrate derived from a culture of a different strain of *B. typhosus* (T_{240}). The culture was made in tryptic digest broth in the usual manner. Rabbits 26-5 and 26-6 were injected into the upper and lower right and left areas of the skin of the abdomen respectively with unheated filtrate T_{240}, with T_{240} filtrate heated to 60°C. for 1 hour, with T_{240} filtrate heated in the Arnold steam sterilizer for 1 hour and the T_{240} filtrate heated in the autoclave for 45 minutes. 24 hours later the rabbits were injected intravenously with the unheated filtrate T. D. T_{240}. The dose was 2 cc. per kilo of body weight. 4½ hours after the intravenous injections all the 4 prepared areas showed extremely severe hemorrhagic reactions which varied in size from 2 × 2 cm. to 4 × 3 cm.

Further attempts were made to inactivate the skin preparatory factors of T. D. T_{210} filtrates by autoclaving:

The filtrates were first diluted 1:2 and then autoclaved for 45 minutes. In other experiments the autoclaving of diluted and undiluted T.D.T_{240} filtrate was prolonged to 1 hour. In all the experiments the skin preparatory factors remained unaffected by the process.

Protocol 3.—A mixture of equal amounts of filtrates derived from cultures of 4 different strains of *B. typhosus* (Mt. Sinai, L, 215$_A$ and 215$_B$) was employed for these experiments. 6 rabbits were used. The experiment was performed in a manner identical with that described in Protocol 1. 1 rabbit died 1 hour after the injection. No reading was made. 4 rabbits showed very severe hemorrhagic

reactions in the upper and lower right and upper left areas. The lower left areas were entirely negative. 1 rabbit was resistant to the phenomenon of local skin reactivity to *B. typhosus* culture filtrate. The same readings were obtained 24 hours after the intravenous injection.

As is seen from the experiments of this part of the work, the skin preparatory factors of *B. typhosus* possessed considerable resistance to heating. Filtrates derived from certain strains lost these factors when autoclaved for 45 minutes. There was encountered, however, a filtrate of one strain (T_{240}) which resisted autoclaving for 1 hour.

7. The Effect of Different Hydrogen Ion Concentrations upon the B. typhosus Skin Preparatory Factors.

It was desirable to determine the effect of acid and alkali upon the skin preparatory factors of *B. typhosus* and, incidentally, to determine whether there is a difference in the heat resistance of filtrates adjusted to various pH.

The experiments were performed as follows:

The *B. typhosus* culture filtrate T. D. T_L was adjusted under sterile precautions to pH 9.0, 8.6, 7.6, 7.0, 6.6, 5.4 and 4.0.[2] The final dilution of the filtrates of various pH was 1:2. The filtrates of pH 9.0 and 4.0 were used on the day of adjustment; those of pH 8.6 to 6.0 were injected 25 hours after the adjustments. Before use, given amounts of filtrates of various pH were heated for 1 hour at 60°C., in the Arnold sterilizer and in the autoclave. A group of 4 rabbits was employed for experiments with heated and non-heated filtrates of each pH. The upper right areas of the skin of the abdomen were injected with the unheated filtrates. Filtrates heated for 1 hour at 60°C., filtrates heated for 1 hour in the Arnold sterilizer and filtrates heated for 1 hour in the autoclave were injected into the lower right and upper and lower left areas, respectively. The center of the skin of the abdomen was injected with non-adjusted and unheated T. D. T_L filtrate diluted 1:2.[3] 24 hours after the preparatory skin injections the rabbits received intravenously the unheated and non-adjusted T. D. T_L filtrate. The dose was 3 cc. per kilo of weight. The susceptible animals reacted severely 5 hours after the intravenous injections. Reactions were obtained in upper and lower right and upper left areas. The lower left areas were entirely negative. The same readings were made 24 hours after the intravenous injection.

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[2] No buffers were used for adjustments. ·
[3] The pH of the filtrate was 7.9 to 8.0.

As is seen from these experiments, there was no inactivation of the skin preparatory factors at a hydrogen ion concentration range of from 9.0 to 4.0 and there was no change in the heat resistance at the various pH. The factors derived from the strain used (T. D. T_L) were invariably inactivated by autoclaving for 1 hour.

8. Incubation Period in Preparation of the Skin to the Phenomenon of Local Skin Reactivity to B. typhosus Culture Filtrate.

In the experiments thus far reported there was allowed an interval of 24 hours between the skin preparatory injections and the intravenous injection of *B. typhosus* culture filtrate. The following experiments were made in order to determine the optimum interval between the injections necessary to elicit the reactivity:

Protocol 1.—6 rabbits (Nos. 48-2 to 48-6) were injected into 4 areas of the skin with T. D. T_L filtrate. Rabbits 48-2, 48-3 and 48-4 received intravenous injections of the filtrate 2 hours after the skin injections. The dose was 3 cc. per kilo of weight. No reactions were seen in these rabbits in the course of the following 48 hours.

Rabbits 48-5, 48-6 and 48-7 were injected intravenously with 3 cc. per kilo of body weight 48 hours after the skin injections. The skin remained unchanged for 48 hours later.

No definite conclusions could be drawn from this experiment, since as shown on page 251 a certain percentage of animals was spontaneously resistant to the phenomenon of local reactivity to *B. typhosus* filtrate. In order to eliminate this objection, the following experiment was performed:

Protocol 2.—The upper right areas of the skin of the abdomen of Rabbits 1-7, 1-8, 2-0, 2-6, 2-7 and 43-9 were injected with 0.25 cc. of T. D. T_L filtrate. 24, 48 and 55 hours later 0.25 cc. of the same filtrate was injected into the lower right, upper left and lower left areas of skin of the abdomen respectively. 56 hours after the first skin injections filtrate T. D. T_L was injected intravenously into these rabbits. The dose was 3 cc. per kilo of body weight. The morning following the intravenous injections namely 15 hours after the intravenous injections the following results were obtained: Rabbit 1-7 was found dead and no reading was possible. Rabbits 1-8, 2-0, 2-6, 2-7 and 43-9 showed no reactions in the upper right and lower left areas. Both lower right and upper left areas showed very pronounced hemorrhagic reactions which varied in size from 1 × 1 to 2 × 2 cm.

From these experiments the following could be concluded: For the reproduction of the described phenomenon a definite interval of time was required between the preparatory skin injection and the intravenous injection of the filtrate.

No definite data were available as yet in reference to the exact number of hours required to allow for the preparation of the skin. It could only be stated that 2 hours were insufficient and that the state of reactivity did not last longer than 32 hours.   The phenomenon could be invariably reproduced if an interval of 24 hours was allowed between the skin and intravenous injections.

In Protocol 2 it will be noticed that skin reactivity appeared 8 hours after the skin preparatory injection.   In these animals, however, the reading of the skin reaction was made 15 hours following the intravenous injections, instead of after the customary 5 hours.   The significance of this observation will be considered in a subsequent report.

9. Local Reaction to Repeated Skin Injections of B. typhosus Culture Filtrates.

In the work described up to this point severe hemorrhagic and necrotic local reactions were obtained by skin-intravenous injections. Although no attempts were made as yet to study the mechanism of the phenomenon, it was of interest to determine whether the intravenous route was essential for the reaction.   The following protocol illustrates these attempts:

Protocol 1.—Rabbits 16-0, 16-1 and 16-2 received each 4 skin injections of T. D. T_L filtrate into the usual areas.   0.5 cc. was injected into each area.   No reactions were seen in these rabbits 24 hours after the injections.   The same areas were then reinjected with the filtrate.   The amount of filtrate was again 0.5 cc. for each area.   The readings were, then, made every hour for the following 6 hours and again 20 hours later.   About 1 to 2 hours after the reinjections there appeared reddening and swellings in the 4 areas.   The reddening became very pronounced towards the end of 5 hours (4+ erythema).   Rabbit 16-1 was killed and sections of the inflamed skin were made.   The following morning Rabbits 16-0 and 16-2 showed reactions of the same type as the day before.   They were then given intravenous injections of T. D. T_L filtrate in a dose of 3 cc. per kilo of body weight. 4 hours after the intravenous injections both rabbits showed very extensive hemorrhagic necrotic reactions which varied from 2×3 to 3×4 cm. in size.

The skin section of Rabbit 16-1 showed inflammatory changes.   There was an

infiltration of polymorphonuclear neutrophil leucocytes. The leucocytes did not appear necrobiotic. There was no rupture of the blood vessels. No thrombi were found. Some dilatation of the blood vessels was seen in places. The experiment was repeated on 8 more animals with identical results.

As is seen from this experiment repeated skin injections of the filtrate with a 24 hour interval between the injections did not result in the hemorrhagic and necrotic type of reaction described in this paper. The type of the reaction produced by repeated skin injections consisted of a pronounced cellular infiltration, but in contrast to the phenomenon under consideration, there was no breaking up of the cells and no severe damage to the blood vessels. Moreover, the susceptibility of the animals to the hemorrhagic type of reactions was demonstrated, for, 24 hours after the second skin injections, intravenous injections of the *B. typhosus* filtrate were given and there developed severe hemorrhagic and necrotic reactions at the site of previous skin injections. It seems, therefore, that the preparatory skin injections have to be followed by injection of the filtrate through the intravenous route for the reproduction of the phenomenon described.

Further work on the mechanism of the reaction is under progress. It is intended also to amplify the experiments on repeated skin injections with toxic filtrates of different strength.

CONCLUSIONS AND SUMMARY.

A phenomenon of local skin reactivity to *B. typhosus* culture filtrates is described in this report. The reactivity was induced by skin injections of the filtrate followed 24 hours later by an intravenous injection of the same filtrate. The local response consisted of severe hemorrhagic necrosis and was fully developed 4 to 5 hours after the second injection.

About 78 to 79 per cent of the rabbits employed were susceptible to this phenomenon.

Different areas of the skin of the abdomen, when similarly treated, responded with equal severity to the intravenous injection. There were variations in the size of different areas in the same animals.

The intensity and size of the local hemorrhagic reactions were not related to the intensity of the erythema produced by the preparatory skin injections. Following intravenous injection very severe hemor-

rhagic reactions were obtained, in those areas which reacted negatively in this respect to the preparatory skin injections.   Evidently, the local trauma produced by the preparatory skin injections was not responsible for the localization of the toxic factors introduced by the intravenous route.

It was necessary to allow a short interval of time between the skin preparatory injections and the intravenous injection, for the reproduction of the phenomenon.   An incubation period of 2 hours was insufficient.   An interval of 24 hours was invariably sufficient.   The ability to react disappeared in 48 hours after the preliminary skin injections.

Repeated direct injections of the filtrate into the same areas of the skin, with an interval of 24 hours between the injections, did not result in reactions similar to the above described hemorrhagic necrosis.   The second skin injection was followed by reddening, some swelling and a local accumulation of polymorphonuclear neutrophil leucocytes which showed no signs of necrobiosis.   There was no rupture of blood vessels.   Skin injections followed after a suitable interval by intravenous injection, were necessary for the reproduction of the severe local hemorrhagic response.

Skin reactivity to *B. typhosus* culture filtrate injected intravenously was not induced by turpentine in various dilutions, sterile tryptic digest broth, culture filtrates of 4 strains of streptococci or by the *Streptococcus erysipelatis* toxin.

It was possible to titrate the skin preparatory factors.   Dilutions of the filtrate up to 1:64 were able to induce the local skin reactivity. But, whereas dilutions up to 1:4 invariably prepared the skin so that very severe hemorrhagic reactions followed the second injection in susceptible animals, dilutions from 1:8 to 1:64 were uncertain and their preparatory effect varied in different animals.

The skin preparatory factors showed considerable heat resistance. The heat resistance varied with the strains employed.   One strain produced factors totally resistant to heating in the autoclave for 1 hour.   However, there was definite and unquestionable inactivation of these factors as derived from other strains when the filtrate was diluted 1:2 and heated in the autoclave.

Various hydrogen ion concentrations in the range from 9.0 to 4.0 had

no effect upon the skin preparatory factors. Heat resistance was not modified by the various pH within this range.

The mechanism of the phenomenon described has not been fully studied as yet. An experimental comparison of it with the manifestations of bacterial allergy of the skin is necessary. There are certain features, however, which *considered together*, distinguish this phenomenon from the known phenomena of bacterial hypersusceptibility. These features are: local reactivity; the short incubation period necessary to induce the local reactivity; the short duration of the state of reactivity; the ability to induce local reactivity by a single skin injection; the severity of the reaction; and the necessity to make the second injection of the toxic agent by the intravenous route.

Studies on the relation of specific antisera to the phenomenon described are under way.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. 5 areas of the skin were injected each with 0.3 cc. of T. D. T_L filtrate. 24 hours later the rabbit received intravenous injection of the same filtrate. The dose was 2.5 cc. per kilo of body weight. Appearance of reaction at the site of prior skin injections 5 hours after the intravenous injection.

FIG. 2. This rabbit was treated in a manner identical with that represented by Fig. 1. In this rabbit there was a confluent reaction extending from the upper right and lower right areas to the center of the skin of the abdomen.

PLATE 7.

FIG. 3. Hematoxylin-eosin.   × 230.   Section of skin from Rabbit 14-1.   Microscopic appearance of hemorrhagic reaction 5 hours after intravenous injection of T. D. T_L.   Note necrobiosis of white blood cells.

FIG. 4. Hematoxylin-eosin.   × 270.   Section of skin from Rabbit 16-1.   Microscopic appearance of reaction produced by repeated skin injections of T. D. T_L.   No necrobiosis of white blood cells.

FIG. 1.

FIG. 2
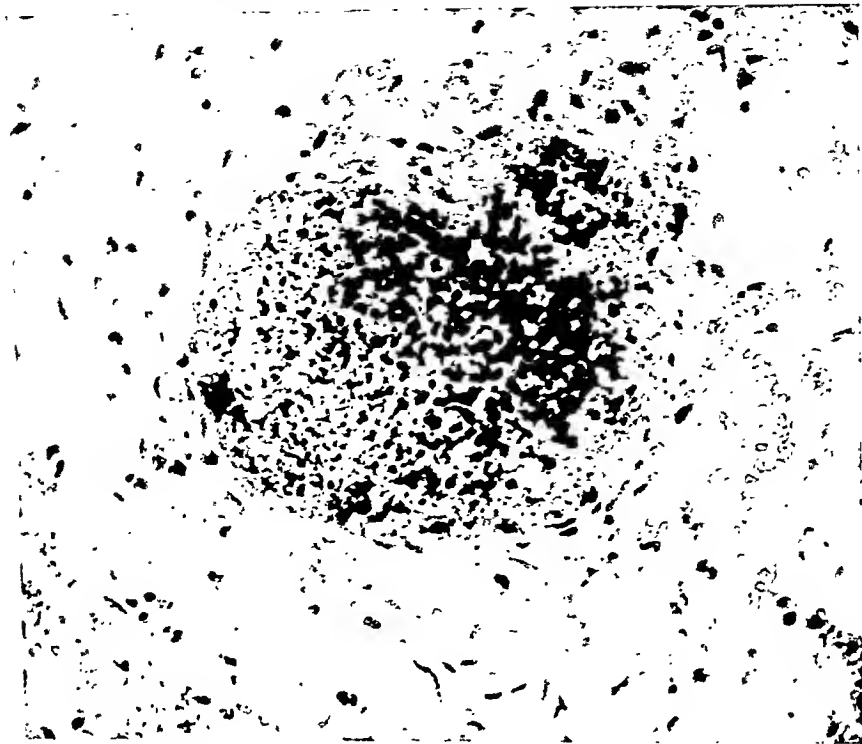
Fig. 3.
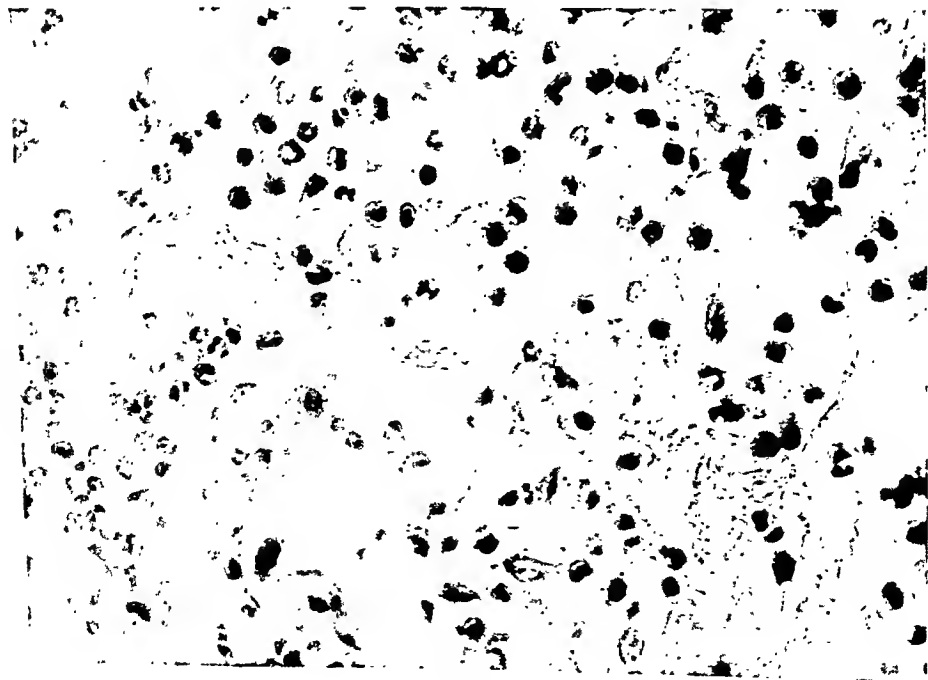
Fig. 4.

(Sahyun et man: *B. typhosus* toxic substances. I.)

STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

IX. Evidence of Hydrolysis of Bacterial Protein during Lysis.

By D. M. HETLER, Ph.D., and J. BRONFENBRENNER, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The disappearance of bacteria and the coincident accumulation of an agent capable of bringing about this disappearance in serial subcultures are the characteristics which originally drew the attention of Twort to the phenomenon which later became known under the name of bacteriophagy, or the phenomenon of d'Hérelle. From the outset, Twort saw three distinct possibilities in interpreting his observations: He thought that the disappearance of the bacteria might be due to their passage into a subvisible state as a stage in a peculiar life cycle initiated under the conditions of the experiment. He saw as an alternate possibility that the disappearance of bacteria was due to rapid autolysis of the cells. Finally, he deemed it possible that bacteria underwent disintegration as the result of an acute infectious disease caused by an extraneous, ultramicroscopic virus.

Each one of these tentative explanations found a number of supporters among those subsequently studying the phenomenon. Our investigations, however, have suggested still another explanation of the appearances, namely, that they are due to the rupture of the bacterial cell wall which is unable to withstand the rise in internal pressure caused by the imbibition of water. The fact that the cytoplasm is quickly dissolved in the surrounding medium after the bursting of the bacteria indicates that the intracellular contents have been rendered soluble through some process of digestion prior to the bursting. Granting this to be true, the lysed cultures should show the presence of the products of digestion of the bacterial cytoplasm. Attempts have been made unsuccessfully in the past to find the products of hydrolysis of bacterial protein in lysed cultures. It seems possible that the failure was due to the fact that lysis was

carried out in beef infusion broth—a medium so rich in various protein-split products that a small increase caused by the hydrolysis of bacterial protein might have been masked.   In our experiments here to be reported the lysis was made to take place in synthetic media, free from organic nitrogen.

EXPERIMENTAL.

Media.—Since the lysis of bacteria is conditioned by their free multiplication, it was first necessary to find synthetic media in which the bacteria which we intended to use, namely, *B. coli*, *B. pestis caviæ*, and a thermophilic bacillus respectively, would grow freely, and in which typical lysis would take place upon the addition of phage.   After several trials we came upon two such media.   The first, which represents a slight modification of the synthetic medium of Pozerski, and which was found to be suitable for *B. coli* and *B. pestis caviæ*, is prepared as follows:

Saccharose..	20.0 gm.
Magnesium sulfate......................................	1.0 "
Potassium sulfate.......................................	1.0 "
Disodium phosphate (anh.)............................	1.0 "
Ammonium succinate...................................	6.0 "
Distilled water...	1000.0 cc.

The pH is adjusted to 7.3 before sterilization and the medium is autoclaved at 15 pounds pressure for 15 minutes.   After sterilization the pH varied from 7.0 to 7.1.

The thermophilic bacillus was grown in a medium similar to the one proposed by Frankel, Barber, and Pyle,[1] the composition of which is:

Ferric chloride.......................................	0.001 gm.
Magnesium sulfate......................................	0.001 "
Calcium chloride.......................................	0.001 "
Dextrose...	10.000 "
Disodium phosphate (anh.).........................	7.000 "
Dipotassium " "	9.000 "
Ammonium acetate......................................	1.400 "
Distilled water...	1000.0 cc.

The pH is adjusted to 7.0 with 10 per cent phosphoric acid.   Sterilization is by autoclave at 15 pounds pressure for 15 minutes.

Chemical Methods.—After a suitable period of growth, all cultures were subjected to the same treatment.   20 gm. of hydrated barium hydroxide were added to each liter of medium.   The volume of the liquid was reduced to 100 cc. by distillation under diminished pressure from a water bath kept at a temperature

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[1] Frankel, Barber, and Pyle, *J. Infect. Dis.*, 1921, xxiv, 9.

between 60° and 70°C. At this point the material was tested for ammonia by Nessler's reagent. If ammonia was still present, sufficient 5.0 per cent barium hydroxide solution to give a slight excess of alkali together with 500 cc. of distilled water was added, and the distillation was continued until the Nessler's reagent gave a negative test. The precipitate of barium salts was then removed by filtration, and the precipitate thoroughly washed. The barium remaining in the filtrate and washings was removed by carefully adding dilute sulfuric acid. The reaction was made slightly alkaline to litmus by dilute sodium hydroxide, and again distilled to a volume of 100 cc. The precipitate of barium sulfate was removed by filtration or by centrifuging and then washed. The filtrate and washings were distilled as before and washed into a 50 cc. volumetric flask, to

TABLE I.

	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino N}_2}{\text{Total N}_2}$		Increase of $\frac{\text{NH}_2}{\text{N}_2}$ ratio in the presence of phage
	Control	Phage	Control	Phage	Control	Phage	
Column..................	1	2	3	4	5	6	7
	gm.	*gm.*	*gm.*	*gm.*			*per cent*
B. coli...............	0.00248	0.00206	0.000842	0.000842	0.339	0.408	20.3
" "	0.00700	0.00707	0.00155	0.00183	0.221	0.259	17.2
" "	0.00686	0.00700	0.00140	0.00168	0.205	0.241	17.5
" pestis cavi...	0.00945	0.00875	0.00325	0.00553	0.344	0.632	83.7
" " "	0.00794	0.00770	0.00232	0.00383	0.293	0.497	69.7
" " "	0.00472	0.00490	0.00155	0.00291	0.329	0.595	80.8
Thermophile.........	0.00805	0.00770	0.001725	0.001725	0.214	0.224	4.58
"	0.00630	0.00630	0.001390	0.001668	0.221	0.265	20.2
"	0.005425	0.005775	0.000973	0.001390	0.180	0.241	39.6

which sufficient acetic acid had been added to make the solution acid. The total nitrogen in this filtrate was determined on 10 or 20 cc. aliquots by the Kjeldahl method. Amino nitrogen was determined by the method of Van Slyke

General Procedure.

All cultures used in the experiments, as well as the lytic filtrates employed, were carried through a number of passages on appropriate synthetic media previous to their use in the tests described below.

The first series of tests was made with the idea of determining any difference in the free amino acid content between cultures containing phage and those without phage.

TABLE

	18 hr. period. Before addition of phage					
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{Amino\ N_1}{Total\ N_1}$	
	Control culture	Culture to which phage is to be added	Control culture	Culture to which phage is to be added	Control culture	Culture to which phase is to be added
Column	1	2	3	4	5	6
	gm.	gm.	gm.	gm.		
B. pestis caviæ	0.002975	0.002975	0.0006356	0.0006356	0.214	0.214
" " "	0.002800	0.002800	0.0005520	0.0005520	0.197	0.197
" coli	0.001750	0.001750	0.000349	0.0003490	0.199	0.199
" "	0.002275	0.002275	0.0004147	0.0004147	0.182	0.182

TABLE

	18 hr. period. Before the second addition of bacteria						
	Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{Amino\ N_1}{Total\ N_1}$		Increase of the $\frac{NH_2}{N_2}$ ratio in the culture with phage $\left(\frac{Column\ 6-Column\ 5}{Column\ 5}\right)$
	Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage	
Column	1	2	3	4	5	6	7
	gm.	gm.	gm.	gm.			per cent
Experiment I	0.00350	0.00315	0.000281	0.000280	0.0804	0.0889	10.57
" II	0.00263	0.00245	0.000281	0.000351	0.107	0.1430	33.70

II.

24 hrs. after addition of phage. Total of 42 hrs.						Increase of the $\frac{NH_3}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 12-Column 11}}{\text{Column 11}}\right)$	Increase of the $\frac{NH_3}{N_2}$ ratio in the control during second period of incubation $\left(\frac{\text{Column 11-Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_3}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 12-Column 6}}{\text{Column 6}}\right)$
Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino N}_2}{\text{Total N}_2}$				
Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
7	8	9	10	11	12	13	14	15
gm.	gm.	gm.	gm.			per cent	per cent	per cent
0.004725	0.00560	0.001400	0.00203	0.2963	0.3625	22.3	38.4	69.4
0.005950	0.00377	0.001242	0.001656	0.2087	0.2867	37.3	10.6	45.2
0.005770	0.00245	0.001576	0.000685	0.2729	0.2797	2.4	37.1	40.5
0.005075	0.00245	0.001380	0.000828	0.2719	0.3379	24.3	49.4	85.6

III.

6 hrs. after the second addition of bacteria. (Total period 24 hrs.)						Increase of the $\frac{NH_3}{N_2}$ ratio in the culture with phage $\left(\frac{\text{Column 13-Column 12}}{\text{Column 12}}\right)$	Increase of the $\frac{NH_3}{N_2}$ ratio in the control during the second period of incubation $\left(\frac{\text{Column 12-Column 5}}{\text{Column 5}}\right)$	Increase of the $\frac{NH_3}{N_2}$ ratio in the presence of phage during second period of incubation $\left(\frac{\text{Column 13-Column 6}}{\text{Column 6}}\right)$
Total nitrogen per 1000 cc. of culture		Amino nitrogen per 1000 cc. of culture		Ratio: $\frac{\text{Amino N}_2}{\text{Total N}_2}$				
Control culture	Culture with phage	Control culture	Culture with phage	Control culture	Culture with phage			
8	9	10	11	12	13	14	15	16
gm.	gm.	gm.	gm.			per cent	per cent	per cent
0.00455	0.00420	0.000557	0.000697	0.1224	0.1658	35.4	52.1	86.5
0.00500	0.00612	0.000826	0.001380	0.147	0.202	37.4	37.4	41.2

In each experiment two flasks of 1 liter each, containing precisely the same medium, were used, and each was inoculated with 1 cc. of an 18 hour culture of bacteria grown in the synthetic medium. To one of the flasks was added 0.5 cc. of phage, also grown on synthetic medium—the phage titer varying between 10^{-7} and 10^{-9}. The other flask served as a control.

The cultures of B. coli and B. pestis caviæ were incubated for 40 to 48 hours at 37°C., while the thermophilic bacillus was grown for 24 hours at 48°C. After the period of growth the cultures were treated as previously described.

Table I gives the results of these experiments. A comparison of Columns 5 and 6 shows that the amount of amino acids in the cultures containing phage is much larger than that in the control cultures.

The direct source of the increase in amino nitrogen in the presence of phage is not immediately apparent. The accumulation of amino nitrogen may be the result of increased hydrolysis of bacterial protein. It is possible, however, that, owing to a marked diminution in the numbers of live bacteria resulting from active lysis, the utilization of amino nitrogen present in the medium as the result of normal autolysis, is much slower in the flask containing phage than in the control. Such a condition would also lead to the accumulation of amino nitrogen in the flask with phage.

To determine the point thus brought up, the following experiment was performed:

Two flasks, each containing 2 liters of medium, were inoculated with 2 cc. of an 18 hour culture of bacteria and incubated for 18 hours. At this time 1 liter of the culture was removed from each flask for analysis (Table II, Columns 1 to 6), 1 liter of culture being retained in each flask. To one of the flasks 10 cc. of purified (amino nitrogen-free) bacteriophage were added; the other flask received 10 cc. of the same phage inactivated by heat and served as a control. After a further period of 24 hours of incubation at 37°C., the contents of both flasks were analyzed (Table II, Columns 7 to 12).

From a comparison of the data recorded in Table II (Columns 5 and 11), it appears that in the absence of bacteriophage, the con-·centration of amino nitrogen continues to increase on prolonged incubation of cultures. Thus (Column 14), if the effect of phage was merely to bring about destruction of bacteria, the result would have been to lower the rate of accumulation of amino nitrogen. The actual observation (see Table I, Columns 5 and 6, and Table II, Columns

11 and 12) shows, however, that in the presence of active lysis, amino nitrogen accumulates in the culture containing the phage considerably faster than in the control culture (compare Columns 14 and 5). These findings indicate that phage increases the rate of hydrolysis of bacterial protein.

It was thought that this point could be further elucidated by adding quantities of young, living bacteria to test cultures, and determining the effect upon the amino nitrogen content.

2 cc. of an 18 hour culture of *B. pestis caviæ* were added to each of two flasks containing 2 liters of the synthetic medium. In addition, 0.5 cc. phage (titer 10^{-5}) was added to one of them. After incubation for 18 hours, 1 liter was taken from each and analyzed as before (Table III, Columns 1 to 6). Equal amounts of a heavy suspension of young bacteria in synthetic medium, obtained by centrifuging the organisms from 2 liters of an 18 hour culture, were added to the remaining portions of the test cultures. 6 hours later these cultures were analyzed (Table III, Columns 8 to 13).

By comparing Columns 14 and 7, it is readily seen that after the addition of large numbers of living bacteria, the free amino acid content increases, and the increase is greater in those cultures containing phage. These results strengthen the conclusion drawn from preceding tests, namely, that lysis of bacteria by phage is accompanied by hydrolysis of bacterial protein.

CONCLUSIONS.

1. During the process of lysis by bacteriophage, there is an appreciable increase in the amount of free amino acid present in the culture.

2. The increase of free amino acid is due to hydrolysis of bacterial protein.

STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

X. Toxin Production by Normal and by Phage-Resistant Shiga Dysentery Bacilli.

By RALPH S. MUCKENFUSS, M.D., and CHARLES KORB, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

That the administration of bacteriophage during the course of an infectious disease frequently fails to affect the course of the infection is a common finding. One of the principal reasons assigned for this is the development of a race of bacteria resistant to lysis. Since it is well recognized that the resistant bacteria often show marked divergences from the type in many of their biological characteristics, it becomes of importance to know if their ability to produce disease is also different. There have been few studies of this question.

The commonly accepted view is that resistant bacteria are also characterized by an increased virulence. D'Hérelle (1) makes the general statement that bacteria resistant to the bacteriophage show an enhanced virulence, and then extends this, saying (2) "the degree of virulence of a bacterium is strictly in relation with its degree of resistance to the bacteriophage." Later (3), he states that susceptible *B. coli* is harmless, and that when pathogenic, it is the result of its resistance to phage, but he gives no experimental data in support of this contention.

In studying *B. pestis*, d'Hérelle (4) found that while the susceptible organism kills a guinea pig in a dose of 0.1 cc., the resistant strain kills in a dose of 0.0002 cc. Quiroga (5) obtained a resistant strain of *B. pyocyaneus*, of which a loopful injected intravenously killed in 24 hours, while a loopful of the susceptible strain killed in 3 days. Gratia (6) described a resistant strain of *B. coli* that was highly virulent for guinea pigs, not subject to phagocytosis, and that caused septicemia; while the susceptible strain was weakly virulent, subject to phagocytosis, and caused purulent peritonitis. Kauffmann (7) described resistant (*schleimige*) strains of *B. coli* characterized by greatly increased virulence for rabbits and guinea pigs and by resistance to phagocytosis. Wollstein (8) described a resistant strain of Shiga dysentery bacilli that would kill rabbits in 1/5 the dose required for the susceptible strain. Dutton (9), working with streptococci, says that the

outcome of disease depends upon the balance between the virulence of the bacteriophage and the virulence of the bacterium. He, however, considers all streptococci to be mixed with bacteriophage, so that virulence of the bacteria in this case indicates their ability to survive, and not variation in any other sense.

All of the reported studies were made with suspensions of the bacteria and concerned their ability to kill animals; none was related to toxin production. We therefore undertook the study of toxin production by *B. dysenteriæ* Shiga, an organism that causes disease by the elaboration of toxin and not by invasion.

Olitsky and Kligler (10) described the production of two types of toxin by the Shiga dysentery bacillus. The exotoxin acts on the nervous system, determining a paralysis, while the endotoxin acts on the intestinal tract, causing diarrhea and hemorrhages in the intestinal wall. Their methods were followed in our studies, and while a complete separation of the effects of the two toxins was not usually obtained, the predominance of those of one type over the other, depending on the method of preparation, was ordinarily found. In additional experiments an anti-exotoxin was also used for purposes of separation, but the results did not differ from those secured by other methods, so the protocols are not included. An exact quantitative study of toxin production was not attempted, as it sufficed for the object of the experiments to detect whether loss or great increase of this property had occurred. Only the time of death is recorded in the tables, but not only the symptoms but the autopsy findings were characteristic for the type of toxin used. The tables are each typical of experiments that were repeated several times.

EXPERIMENTAL.

B. dysenteriæ Shiga 109 and Laudman phage, active on it to a titer of 10^{-9}, were used.

Resistant bacteria were isolated by continuing incubation after lysis in broth until an overgrowth of resistant bacteria occurred. This was streaked in a Petri dish, and restreaked each day from single colonies for six generations, and then inoculated on an agar slant. The resistant strain thus secured was shown to be resistant to lysis and free from phage before being used.

Exotoxin was prepared by growing the bacteria in broth for 5 days at 37°C. and then filtering through a Berkefeld V candle. This sterile material was injected intravenously into rabbits (Table I).

The filtrates from the normal and from the resistant cultures resulted in similar symptoms and autopsy findings, and the severity of the effects was roughly proportional to the dose administered. The variation in the time of death was within the ordinary limits of individual variation.

TABLE I.

The Production of Exotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–94 1110 gm.	Normal	0.75	Died in 18 hrs.
No. 3–44 1300 gm.	Normal	0.3	Died in 64 hrs.
No. 3–49 1325 gm.	Normal	0.3	Died in 130 hrs.
No. 3–95 1100 gm.	Resistant	0.75	Died in 29 hrs.
No. 3–46 1400 gm.	Resistant	0.3	Died in 52 hrs.
No. 4–07 3125 gm.	Resistant	0.3	Died in 55 hrs.

TABLE II.

The Production of Endotoxin.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 3–43 930 gm.	Normal	0.75	Died in 18 hrs.
No. 3–48 1175 gm.	Normal	0.3	Dead in 40 hrs.
No. 3–93 710 gm.	Resistant	0.75	Dead in 18 hrs.
No. 3–50 1160 gm.	Resistant	0.3	Dead in 93 hrs.

Endotoxin was prepared by the method of Olitsky and Kligler (10). The 24 hour growth of the bacteria in Blake bottles was washed off in saline solution (15 cc. to each bottle) and incubated for 3 days at 37°C., and then filtered through a Berkefeld V candle. The filtrate

thus secured was heated to 80°C. for 1 hour before intravenous injection. As the results show (Table II), there was no appreciable difference in the production of endotoxin by the normal and by the resistant strains.

After the completion of the first series of experiments, it was found that the resistant organism showed a tendency to revert to susceptibility. This was not evidenced by visible lysis, but by the fact that when a diluted phage was incubated with the bacterium, it would increase in titer. Recourse was then had to growing the resistant bacteria in the presence of phage to prevent reversion. Toxin preparations obtained from organisms so treated had the same effects as those preparations of which the results are recorded in the previous tables.

TABLE III.

Examination of the Resistant Strain.

Bacteria inoculated..........	0	Normal Shiga	Resistant Shiga	Resistant Shiga after 10 daily transfers
Laudman phage. diluted 1–10,000..................	0.1 cc.	0.1 cc.	0.1 cc.	0.1 cc.
Titer before incubation........	10^{-3}	10^{-3}	10^{-3}	10^{-3}
Incubated 48 hrs. Heated to 56°C. for 50 min. Titrated by Appelmans' method.				
Titer......................	10^{-3}	10^{-9}	10^{-3}	10^{-3}

The series of experiments was again repeated, with freshly isolated resistants. After isolating them in the usual manner and demonstrating that they were not subject to visible lysis and did not carry phage, they were incubated with diluted phage and shown to be unsuitable for its increase. As a further control, the strain was carried through repeated transfers in broth and tests showed that it had not reverted sufficiently to serve for the increase of phage. The results of these tests are given in Table III. This eliminates any doubt of the resistance of the bacteria used, but as a further control, the tests were repeated on bacteria taken from each flask just prior to filtration.

Exotoxin was prepared in the manner previously described. *Endotoxin* was prepared according to the method of McCartney and Olitsky (11), the method

being varied by using bacteria grown overnight on the agar surface in a Blake bottle. The growth was suspended in 25 cc. of saline for each bottle. 10 cc. of this were centrifuged and washed once in saline and then suspended in 5 cc. of 1 per cent sodium carbonate. This was sealed in an ampoule, heated to 56°C.

TABLE IV.

Exotoxin Production by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
A 195 1950 gm.	Normal	0.5	Dead in 72 hrs.
A 194 1985 gm.	Normal	1	Dead in 24 hrs.
A 190 1975 gm.	Normal	2	Dead in 48 hrs.
A 193 2050 gm.	Resistant	0.5	Dead in 48 hrs.
A 196 2060 gm.	Resistant	1	Dead in 24 hrs.
A 192 2025 gm.	Resistant	2	Dead in 24 hrs.

TABLE V.

Production of Endotoxin by Bacteria Completely Resistant.

Rabbit No. and weight	Bacteria used for toxin production	Dose	Results
		cc.	
No. 25 620 gm.	Normal	0.05	Dead in 72 hrs.
No. 29 500 gm.	Normal	0.025	Dead in 96 hrs.
No. 26 610 gm.	Resistant	0.05	Dead in 48 hrs.
No. 27 560 gm.	Resistant	0.025	Dead in 72 hrs.

for 1 hour, and incubated overnight. The resulting fluid was very viscous; it was injected intravenously after cultures had shown it to be sterile (Table V).

The results of these experiments (Tables IV and V), as of the previous ones, show that from the standpoint of toxin production, the normal and the resistant bacteria are identical.

DISCUSSION.

The failure of Shiga dysentery bacilli to show an alteration in toxin production with the development of resistance to the bacteriophage is at variance with the commonly accepted view. While an increased virulence has not always been found in such instances, an alteration has usually been reported. Fejgin (12) described three strains of phage-resistant Shiga dysentery bacilli showing a.loss of toxin production to such an extent that 4 to 5 cc. of a 24 hour broth culture failed to produce characteristic symptoms in a rabbit when injected subcutaneously. Blair (13), by the action of bacteriophage, secured two strains of diphtheria bacilli that were atoxic for guinea pigs. Only one of these, however, was resistant to phage. In this laboratory it was found (14, 15) that resistant strains of B. pestis caviæ were avirulent, and that virulence returned with the reversion to susceptibility.   ·

The experimental data presented and the review of the literature show that the relation existing between resistance to bacteriophage and toxin production or virulence is by no means a constant one. Furthermore, it is a well recognized fact that bacteria resistant to phage may show quite pronounced differences from the ordinary in other characteristics. The most logical explanation of these facts seems to be that the properties of resistance to phage, and of toxin production are two independent manifestations of bacterial variation or dissociation. If this is correct, it should be possible, by examining a sufficiently large number of strains, to secure races showing all possible combinations in the degree of these two properties.

SUMMARY.

1. The production of exotoxin and of endotoxin by normal Shiga dysentery bacilli and by strains resistant to Laudman phage was found to be the same.

2. The presence of phage did not alter toxin production by the resistant organism.

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THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

IV. The Malignant Fibroblast of Jensen Sarcoma.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 8.

In a preceding article,[1] it was shown that the malignant fibroblasts of Sarcoma No. 10 of the Crocker Foundation differ from the normal cell type in certain physiological properties which are undoubtedly responsible for their malignancy. In order to ascertain whether these properties are of general significance, an analysis must be made of the behavior of the active element of as many experimental tumors as possible. Some hidden characteristics common to all tumor cells may then become apparent. The purpose of the experiments described in the present paper was to study the fibroblasts of Jensen sarcoma when they are isolated in pure culture, and to compare them with normal fibroblasts and with the malignant cells of Sarcoma No. 10.

Isolation of a Strain of Malignant Fibroblasts from Jensen Sarcoma.

Jensen sarcoma has been studied by many investigators, and its characteristics are well known. A fragment of this tumor, when sectioned and stained, appears to be composed mainly of fusiform cells with large clear nuclei. Between the cells are seen the darker nuclei of macrophages. These macrophages are about one-sixth less numerous than the fibroblasts. The tumor is easily propagated by transplantation to rats, and kills the animals without producing any metastases.

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[1] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, xlviii, 105.

An active strain of Jensen sarcoma was obtained by us from the Crocker Foundation, through the kindness of Dr. Woglom. On January 6, 1928, a few fragments of a tumor freshly extirpated from a rat were cultivated in three D-3 flasks containing a solid medium composed exclusively of diluted chicken plasma. After coagulation, the clot was washed twice with Tyrode solution and the fluid medium introduced. The medium in the first flask consisted of rat serum, in the second of chick embryo juice, and in the third of Tyrode solution. During the first 24 hours of incubation, the tissue fragments surrounded themselves with macrophages, but these cells were present in a much larger number around the tissues cultivated in serum than around those cultivated in embryo juice. After a few days, the cultures made in embryo juice were composed almost exclusively of fibroblasts, and the area covered by the migrating cells was not very extensive. From the tissues cultivated in rat serum and in Tyrode solution, a large number of macrophages and fibroblasts migrated. After about 10 days, the cultures were transferred to other flasks and an attempt was made to isolate a strain of fibroblasts and a strain of macrophages by cultivating the tissues in different media. One of the media was composed exclusively of rat serum, and the other of chick embryo juice. Most of the tissues fed exclusively on embryo proteins died. The fragments cultivated in rat serum, on the contrary, grew actively, and after about a month a pure strain of cells resembling fibroblasts was obtained. The wandering cells completely disappeared. Each colony of fibroblasts was allowed to grow for about 8 or 10 days in the solid medium. Then the coagulum was removed from the flask and the tissues were extirpated and embedded in a fresh coagulum in another flask.

Inoculations into rats of some of the pure cultures of fibroblasts during February, March, and April, 1928, were followed in about 10 days by the appearance of a small tumor which grew rapidly later. The fibroblast-like cells were obviously malignant.

Morphological Characteristics of the Malignant Cells.

The cytological study of the cultures has been made according to the technique described in a previous article.[2] The colonies are composed

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1926, xliv, 261.

of spindle-shaped cells, with a large nucleus and a refringent cytoplasm. Instead of sending forth fan-like processes, as normal and sarcomatous fibroblasts often do, these cells are strictly fusiform, and rarely present any lateral branches (Fig. 1). Their size is equal to that of normal fibroblasts. The surface of the cells averages 1,456 sq. μ, and that of the nucleus 205 sq. μ. The nucleus is broad and short, and contains one or two nucleoli. The segregation apparatus is composed of small, dust-like vesicles. No degeneration vacuoles were observed. The mitochondria are slightly shorter and larger than those of normal cells. There is no abnormal mitosis. Very few binucleate cells are seen. None of the nuclear or cytoplasmic structures show any evidence of degeneration. There are no dead cells in the cultures. It is evident that this malignant element of Jensen sarcoma is a healthy cell.

Architecture of the Colonies.

The malignant cells congregate in a tissue which is not very dense. They have some tendency to scatter. Their processes do not form an intricate network as in the colonies of normal and Sarcoma No. 10 fibroblasts. The appearance of the tissue is loose. The Jensen colonies are typically fibroblastic in structure, although they may slightly resemble certain communities of macrophages. They are round or oval in shape, less extensive, and more transparent than those of Sarcoma No. 10. From the outer edge of the tissue, many cells wander freely into the surrounding medium. However, they remain bipolar and fibroblast-like, and never assume the appearance of macrophages. From the point of view of their texture, the colonies vary in some measure according to the nature of the medium. In rat serum, they are denser than in chick embryo juice or in calf liver digest. After several months of life in chick embryo juice, some cultures have become thicker and assumed an appearance practically similar to that of Sarcoma No. 10.

Residual Growth Energy.

The residual growth energy of Jensen fibroblasts was tested by the duration of the life and the extent of the growth of the colonies in a

medium composed of Tyrode solution (Text-fig. 1). Four experiments were made, as summarized in Table I. The duration of the life
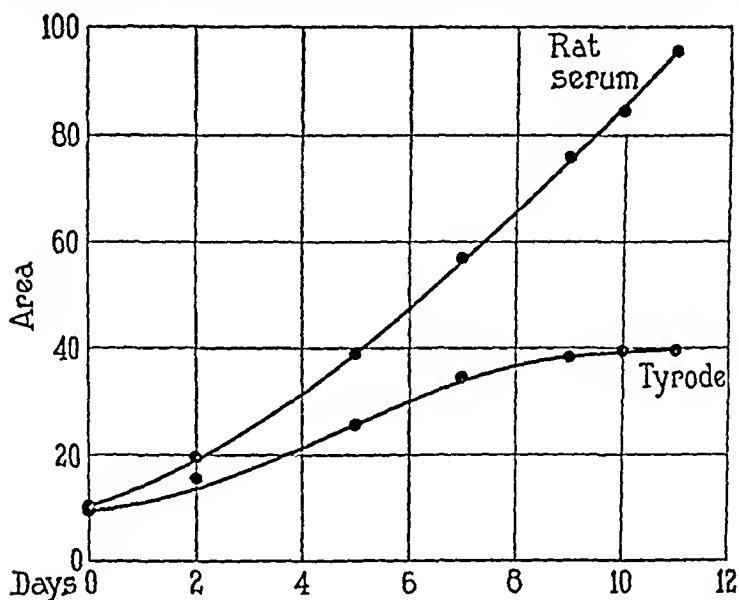
TEXT-FIG. 1. Experiment 6855-C. Residual activity of Jensen sarcoma fibroblasts.

TABLE I.

Residual Energy of Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of life	Relative increase
		days	
1	6855-C	10	3.20
2	10356-D	19	2.16
3	10356-D	19	2.43
4	10356-D	19	4.32
Average..............................		16.8	3.03

of colonies in Tyrode solution did not exceed 17 days. The relative increase was 3. It appears that Jensen sarcoma cells have a little more capacity for storing food than normal cells.

Rate and Duration of the Growth.

The rate of growth of the strain was ascertained in the usual way by measuring the area of the colonies cultivated in a nutrient medium.[3] In Table II, the results of fifteen experiments are summarized, where the relative increases in nutrient media during a passage of several days were compared for 6 to 17 days in the course of a few passages. The area of the colonies may increase about five times in 6 days. The rate of growth varies a great deal, depending on the previous condition of the strain and some other factors. The duration of the life of the tissue in a nutrient medium would appear to be unlimited. After 4 months of life *in vitro*, the malignant fibroblasts proliferate as actively as at the beginning of their cultivation. The inoculation of such cultures into rats produces a tumor in a few days. Therefore, it seems probable that they can live indefinitely *in vitro* without losing their malignancy.

Action of Jensen Fibroblasts on Their Medium.

1. Liquefaction of the Fibrin.—When sarcomatous cells were cultivated in washed chicken plasma, no digestion of the coagulum ever occurred. The medium remained homogeneous. When cultivated in rat plasma, the cells digested the coagulum, as happened in the cultures of Sarcoma No. 10 and of Rous sarcoma.

2. Acid Production.—Fragments of fresh tumor and of fibroblasts in pure culture were embedded in diluted chicken plasma, coagulated with embryo juice, and stained with phenol red, as with Sarcoma No. 10. After a few hours, the sarcomatous colonies appeared as golden yellow spots on the pink background of the medium.

Food Requirements.

Jensen fibroblasts multiplied readily in rat serum (Table II, Text-fig. 2). Colonies have been kept in a mixture of rat serum and Tyrode solution for 4 months, and are still growing with unabated activity. They did not develop in chicken serum (Table III, Text-fig. 3). When the malignant fibroblasts were placed in chick embryo juice, they grew

[3] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.
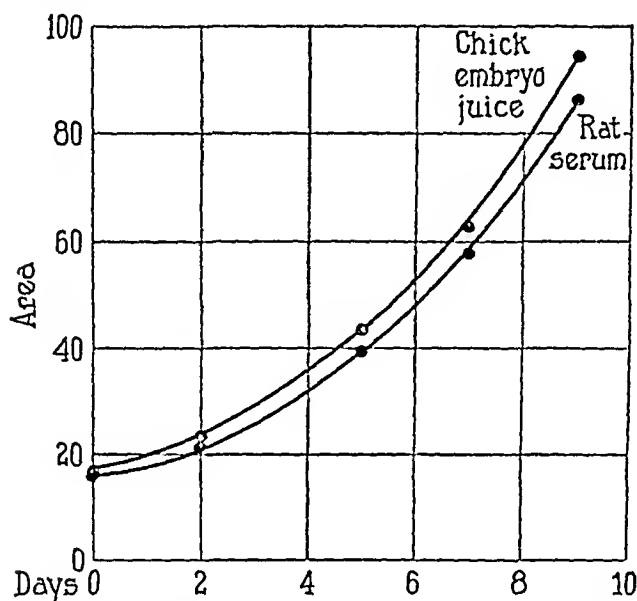
TEXT-FIG. 2. Experiment 10322-D. Effect of rat serum and chick embryo juice on Jensen sarcoma fibroblasts.

TABLE II.

Effect of Rat Serum and Chick Embryo Juice on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	No. of passages	Relative increase during last passage		Ratio: $\frac{E}{C}$
				Control in rat serum	Experiment in chick embryo juice	
		days				
1	10322-D	9	1	4.18	4.65	1.11
2	10322-D	9	1	4.52	4.70	1.04
3	10370-D	17	2	2.20	2.50	1.01
4	10370-D	17	2	3.00	3.20	1.07
5	6688-C	7	1	5.10	5.40	1.06
6	234-J	8	1	3.70	4.90	1.32
7	10364-D	17	2	1.80	1.85	1.04
8	10439-D	7	1	2.54	2.45	0.96
9	10439-D	7	1	2.85	2.76	0.97
10	1691-H	6	1	5.14	5.30	1.03
11	10327-D	17	2	4.44	4.00	0.90
12	10327-D	17	2	4.00	3.46	0.87
13	10380-D	9	1	4.35	3.80	0.87
14	10421-D	23	3	1.20	1.44	1.20
15	10421-D	23	3	1.00	1.00	1.00

more slowly.   In some cultures, the growth stopped altogether and the
colonies died.   However, the tissue became accustomed to the
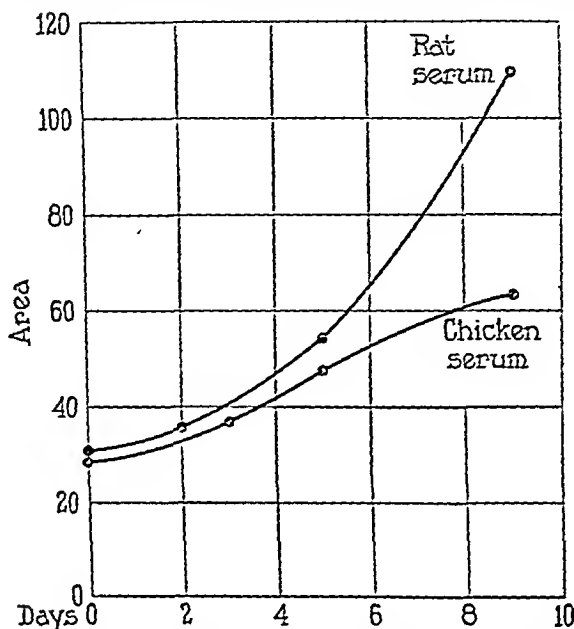
Text-Fig. 3.   Experiment 10426-D.   Effect of rat serum and chicken serum on
Jensen sarcoma fibroblasts.

TABLE III.

Effect of Rat Serum and Chicken Serum on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	Control in rat serum	Experiment in chicken serum	Ratio: $\frac{E}{C}$
		days			
1	10375-D	10	3 80	1.10	0.29
2	10375-D	10	4 00	3.70	0.93
3	10426-D	9	3 57	1.20	0.34
4	10427-D	9	2 57	1 03	0.40
Average.		9 5	3 49	1 76	0.49

medium, and finally grew as actively in embryo tissue juice as in serum.
A comparison was made of the rate of growth of the malignant fibro-

blasts in diluted embryo juice and in rat serum (Table II, Text-fig. 2). Calf liver digests, prepared according to a technique previously de-
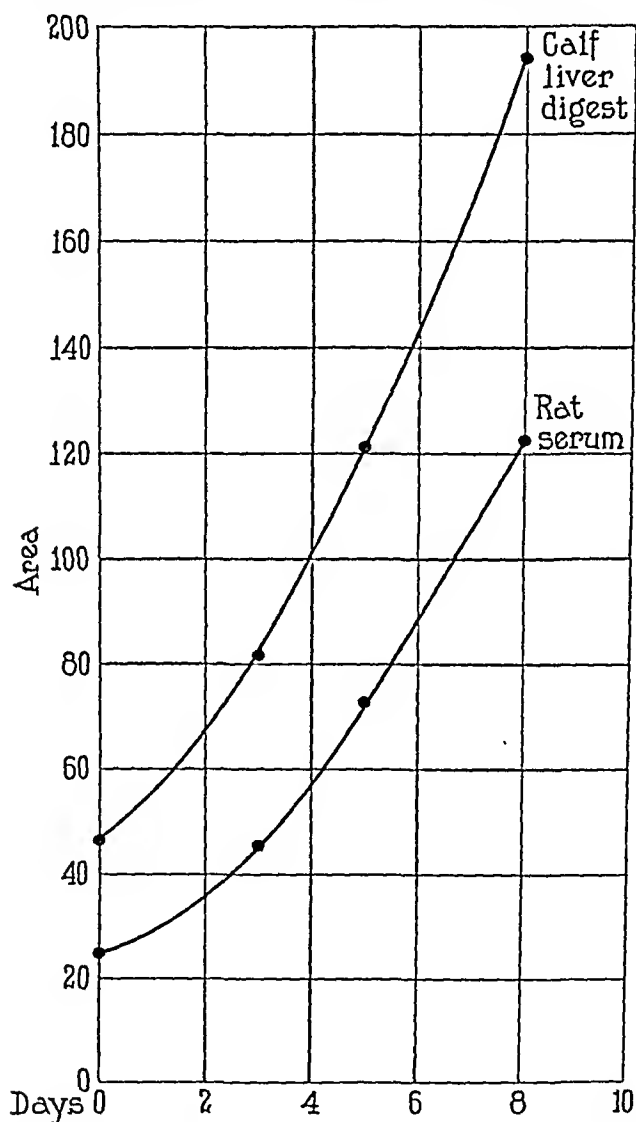
TEXT-FIG. 4. Experiment 348-J. Effect of rat serum and calf liver digest on Jensen sarcoma fibroblasts.

scribed,[4] were used at concentrations of 1:8 and 1:32 as a food for Jensen colonies. No marked differences were observed in the effect

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

TABLE IV.

Effect of Rat Serum and Calf Liver Digest (1:32) on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	No. of passages	Relative increase during last passage		Ratio: $\frac{E}{C}$
				Control in rat serum	Experiment in calf liver digest	
		days				
1	10345-D	10	1	4.40	5.00	1.14
2	10345-D	10	1	3.60	5.80	1.61
3	10345-D	10	1	3.40	5.50	1.62
4	289-J	8	1	3.08	9.99	3.24
5	288-J	8	1	4.44	8.60	1.94
6	10398-D	19	2	2.64	4.80	1.82
7	10398-D	19	2	3.00	3.75	1.25
8	10398-D	19	2	1.52	3.64	2.40
9	10398-D	19	2	2.00	4.29	2.15
10	10460-D	29	3	2.73	4.00	1.47
11	10460-D	29	3	2.65	3.09	1.15
12	10460-D	29	3	2.95	3.57	1.21
13	10460-D	29	3	3.05	4.00	1.31
14	10516-D	35	4	1.90	4.00	2.10
15	10516-D	35	4	3.23	5.67	1.76

TABLE V.

Effect of Rat Bone Marrow on Jensen Sarcoma Fibroblasts.

Experiment No.	Culture No.	Duration of experiment	Relative increase		Ratio: $\frac{E}{C}$
			Control without bone marrow	Experiment with bone marrow	
		days			
1	1877-H	8	3.30	3.67	1.11
2	1877-H	8	2.20	3.30	1.50
3	1877-H	8	3.00	4.60	1.53
4*	10366-D	13	2.60	4.33	1.67
Average...		9.25	2.76	3.68	1.45

* Fluid medium composed of rat serum.

of these solutions. The results of one series of experiments are summarized in Table IV. Calf liver digest appears to be a better nutrient medium for sarcoma cells than serum (Text-fig 4). It has supported

their proliferation for a long time.   After 35 days cultivation in such a medium, the sarcoma fibroblasts proliferate with undiminished rapidity.

Effect of Bone Marrow on Jensen Fibroblasts.

A culture of Jensen fibroblasts was divided into two equal parts. The halves were embedded at the opposite sides of a D-3 flask in a
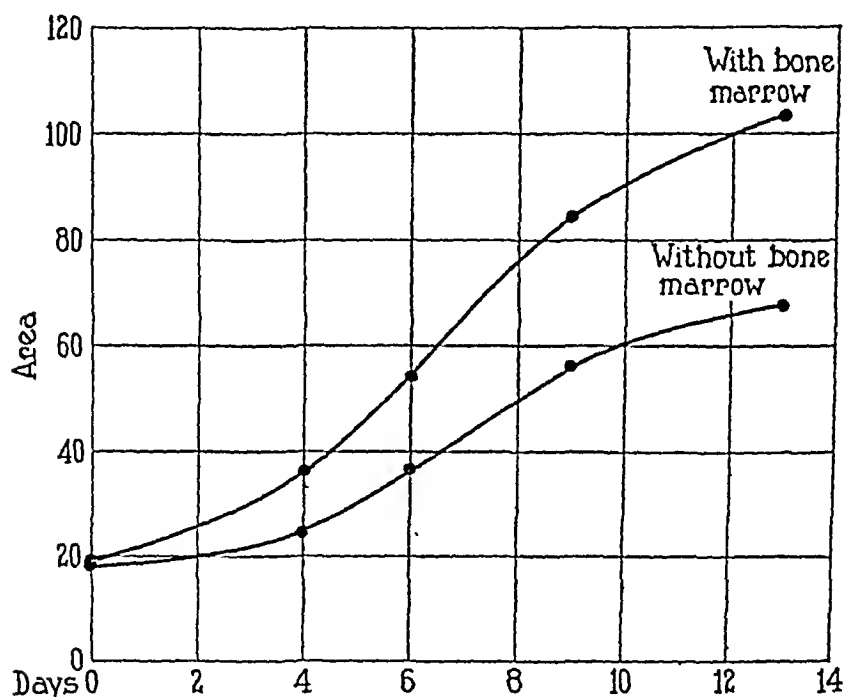
TEXT-FIG. 5.   Experiment 10366-D.   Effect of rat bone marrow on Jensen sarcoma fibroblasts.

coagulum of chicken plasma.   Close to one of the fragments was placed a bit of rat bone marrow.   The fluid medium consisted of Tyrode solution in three experiments, and of rat serum in one.   The measurement of the growth of both colonies of fibroblasts was made according to the ordinary technique.[3]   The results of the experiments, summarized in Table V, show that the presence of the wandering cells from the bone marrow definitely activated the multiplication of the Jensen fibroblasts (Text-fig. 5).

DISCUSSION.

A pure strain of fibroblast-like cells, obtained from a fragment of Jensen sarcoma, has maintained its malignancy unimpaired after several months of life *in vitro*. The element which is the carrier of the malignant characteristics appears to be a true fibroblast, from a morphological point of view. It differs from the rat fibroblasts observed in pure cultures by the refringement aspects of the cytoplasm and its coarseness. The cell is generally bipolar and does not send out any fan-like processes (Fig. 1). Instead of being long and slender as in the normal cells, the nucleus is more globular. While the projected areas of the cell and nucleus of the normal type are respectively 1,960 and 189 sq. μ, those of the Jensen cell are 1,456 and 205 sq. μ. The size of the nucleus is relatively larger in the neoplastic than in the normal cell. The small segregation apparatus is composed of dust-like vesicles and a large number of very small fat granules. No cytoplasmic inclusions or degeneration vacuoles are observed. The mitochondria are shorter and larger than those of normal fibroblasts.

This cell is smaller than the malignant element of Sarcoma No. 10, which has a total area of 2,300 sq. μ, and a nuclear area of 230 sq. μ. Neither malignant cell shows any abnormality. They are healthy elements. The morphological characteristics, by which the specific properties of the cells may express themselves, have not as yet been discovered. It is probable that such characteristics will be detected when more elaborate techniques are used in the morphological investigation of the nucleus and the cytoplasm.

There is a striking difference between the Jensen sarcoma fibroblasts in pure cultures, and the elements composing this same tumor under the circumstances in which it has been studied by other workers. In order that the discrepancies in the results of the investigations may be correctly interpreted, it must be remembered that previous studies of Jensen sarcoma have been made on tissues just removed from the animals, or on fragments of tissues "cultivated" *in vitro* with the technique developed 15 years ago. According to this early procedure, which is still favored by many experimenters in this country and in Europe, a fragment of tissue is placed on a cover glass in a drop of plasma, and, under conditions that are uncontrolled and unknown, it

survives for a few days and degenerates.   In this manner, Fell and Andrews,[5] after a careful cytological study of Jensen sarcoma, have recently described a number of abnormalities and degenerative phenomena, such as binucleate and multinucleate cells and abnormal mitoses.   Similar observations had previously been made on sarcoma tissues by Lambert and Hanes.[6]   Fell and Andrews[5] noted enlarged centrospheres, similar to those described by Lewis[7] in degenerating mesenchyme cells.   They also observed epithelioid cells which differed from the fibroblast-like cells and from the wandering elements, and cells too that were greatly enlarged.   These giant cells obviously came from the fibroblast-like cells.   They usually contained one or two nuclei.   The authors did not believe that there is any histiogenic relationship between those cells and the macrophage type which they also described.   They supposed that the malignant component may be the macrophage, while the fibroblast represents the stroma element.

These abnormalities are evidently phenomena of secondary importance.   They are not directly connected with the malignant characteristics of the cell, since typically malignant sarcoma fibroblasts, after they have lived for some months in pure culture, do not show any of these peculiarities.   They must be attributed to the conditions which are brought about by the crowding of the cells, the setting free of toxic substances by the tissue itself, etc.   It is evident that the mere observation of a fragment of tumor surviving *in vitro* for a few days, according to the procedure adopted by Fell and Andrews,[5] and other experimenters, is inadequate for ascertaining the characteristics which are specific of any cell type.   For the fact is that the Jensen fibroblasts, properly cultured in the pure state, retain malignancy and do not show any striking morphological abnormalities which can be detected by the techniques used thus far.   However, they differ from the normal fibroblasts by their relatively coarse structure and the special character of their colonies.   The texture of the tissue which they form is not dense, although after a few months of growth *in vitro*

[5] Fell, H. B., and Andrews, J. A., *Brit. J. Exp. Path.*, 1927, viii, 413.

[6] Lambert, R. A., and Hanes, F. M., *J. Exp. Med.*, 1911, xiii, 495; xiv, 129; *J. Am. Med. Assn.*, 1911, lvi, 33, 791.   Hanes, F. M., and Lambert, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1910–11, viii, 113.

[7] Lewis, W. H., *J. Exp. Med.*, 1920, xxxi, 275.

it becomes more compact. The cells have some tendency to scatter as macrophages do. But in spite of this characteristic, their colonies are typically fibroblastic, and do not resemble at all the groups of wandering cells scattered through a plasma coagulum. They may be distinguished at first sight from the colonies of Sarcoma No. 10 fibroblasts because they are less opaque, and their elements are less closely interwoven.

The residual growth energy of the Jensen fibroblasts is a little greater than that of normal fibroblasts and of the cells of Sarcoma No. 10. Their rate of growth in a nutrient medium does not differ markedly from that of these other cells. Like them they multiply *in vitro* to an unlimited degree. They feed upon chick embryo juice and on calf liver digest, as do normal fibroblasts and Sarcoma No. 10 fibroblasts. But they differ notably from both cells in that like macrophages they are able to grow indefinitely in rat serum. It is well known that normal fibroblasts die in a short time in such a medium; and Sarcoma No. 10 cells do not survive more than 10 days in diluted blood serum. The capacity of multiplying when placed in diluted serum is of great importance, as it enables the Jensen fibroblasts to grow within the interstitial lymph of the adult organism without the help of any other cell.

The Jensen fibroblasts differ from the normal type in two other respects. They liquefy coagulated rat plasma and produce a large amount of acid. It appears then that the power of dissolving the fibrin of a coagulum made of homologous plasma, and of producing an abnormal amount of acid, belongs to the malignant element of every sarcoma so far investigated: Rous sarcoma,[8] Sarcoma No. 10,[1] and Jensen sarcoma.

It is reasonable to suppose that the growth of Jensen sarcoma within the body is due to the property possessed by the malignant cells of feeding upon blood serum. Whereas normal fibroblasts do not multiply when in blood serum, the Jensen fibroblasts are enabled to proliferate in such a medium. Their malignancy within the organism may arise from this property. Such a difference in the food requirements of Jensen and normal fibroblasts is probably attributable to the increased acid production and proteolytic power of the tumor cell.

[8] Carrel, A., *J. Am. Med. Assn.*, 1925, lxxxiv, 157.

CONCLUSIONS.

1. A pure strain of fibroblasts has been isolated from the Jensen rat sarcoma. The cells give rise to tumors on transplantation into animals and during several months of life *in vitro* have maintained their malignancy unchanged.

2. The malignant cells are generally coarser and more refringent than normal cells. They possess the cytological characteristics of fibroblasts without showing any morphological abnormality. They can be considered as healthy cells. The texture of their colonies is looser than that of normal fibroblasts and Sarcoma No. 10 fibroblasts. Their residual activity does not differ markedly from the normal. They proliferate unlimitedly in a nutrient medium.

3. They liquefy the fibrin of rat plasma and turn phenol red golden yellow. They do not liquefy the fibrin of chicken plasma.

4. They multiply in chick embryo juice, calf liver digest, and also in rat serum. Their growth activity is increased by the presence of bone marrow.

5. The unlimited growth of Jensen sarcoma within the body may possibly be attributed to the ability of the fibroblasts to maintain themselves upon the substances present in rat serum. This property itself probably depends upon the increased enzyme activity of the cells.

EXPLANATION OF PLATE 8.

FIG. 1. Camera lucida drawing of cells of Jensen fibroblasts.   × 1,600.

PLATE 8.

FIG. 1.

(Corded Disease Eye Dust and macrophage IV.)

RECIPROCAL AGGLUTINATION AND ABSORPTION OF AGGLUTININ TESTS WITH FIFTY-FOUR STRAINS OF HEMOLYTIC STREPTOCOCCI ASSOCIATED WITH AN EPIDEMIC OF PUERPERAL FEVER.

By FRANK L. MELENEY, M.D., HELEN ZAYTZEFF, M.D., HAROLD D. HARVEY, M.D., AND ZUNG-DAU ZAU, M.D.

(*From the Research Laboratories of the Department of Surgery, College of Physicians and Surgeons, Columbia University, New York.*)

INTRODUCTION.

In the late winter of 1927 an outbreak of puerperal fever occurred in the Sloane Hospital in New York City, reaching such proportions that the hospital had to be temporarily closed. In the course of 1 month 24 of 163 parturient women developed puerperal fever and 8 died, a mortality of 33 per cent. The epidemiological and clinical features of that epidemic will be reported elsewhere (1, 2). The purpose of this paper is to present the results of the biological and immunological study of 54 strains of hemolytic streptococci which were found to be associated with the epidemic.

This epidemic, like most others, was well under way before it was recognized as such. Two patients died with symptoms of sepsis from whom no cultures were taken and on whom no autopsies were performed. The third fatal case, at autopsy, showed streptococci in the peritoneal exudate. From the fourth, hemolytic streptococci were obtained by culture from the peritoneum after death. Thereafter it was recognized that an epidemic of hemolytic streptococcus puerperal fever was in full swing and cultures were made from the vaginæ and blood of all patients showing a post partum temperature above 101°F. Where death occurred cultures were also made at autopsy. Outside help was called upon to investigate the epidemic and the bacteriological research laboratory of the Department of Surgery undertook the study. There was some delay in organizing the work and it was not

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until 18 days after the delivery of the first case of the series that a systematic bacteriological survey of the hospital was begun in an effort to discover if possible, the source of the infection. In the meanwhile a large group of nurses, one doctor and several patients had left the hospital, so that it was recognized that the survey could not be complete. In this survey it was found that the only places where the hemolytic streptococci could be found with any degree of constancy were the vaginæ of the patients and the noses and throats of some of the attending staff. The laundry, air and various objects in the delivery rooms and wards, the solutions and supplies yielded no hemolytic streptococci in culture. The noses and throats of the patients were entirely negative during and for some time after the epidemic. Altogether 54 strains were cultured, chiefly from the vagina, peritoneum and blood of patients and from the noses and throats of staff members. Two of these strains came from the vaginæ of patients not showing clinical symptoms of puerperal fever. These were picked up during the course of our routine examinations. All of the strains were studied with the view to determine if possible the biologic identity of two or more of these strains.

Krumwiede, Cooper and Provost, in their monograph on absorption of agglutinin (3), have clearly shown that this test is required to establish close biological similarity of bacterial antigens. Heterologous organisms will frequently agglutinate in a serum out of which they cannot completely absorb the agglutinin. The phenomenon of agglutination in such cases is supposed to be due to a group agglutinin. Heterologous strains which absorb all of the agglutinin out of a serum are much closer antigenically to the homologous strain than those which only absorb out the group agglutinin. In order to demonstrate that two organisms are exactly the same antigenically it is necessary to produce in animals an antiserum for each strain and then show that both organisms will not only agglutinate to the same degree in both sera but that they will both, in the same absorptive dose, completely absorb the agglutinin out of both sera. In other words there must be complete reciprocal agglutination and complete reciprocal absorption of agglutinin. Such a relationship brings very strong evidence in favor of the common origin of such strains, and probable sources of infection may be determined by this method. By this test, 2 years

ago, one of us, with Stevens was able to show that the last case in a series of hemolytic streptococcus wound infections was caused by an organism which was antigenically identical with a strain cultured from the unmasked nose of the instrument nurse assisting at that operation. It was further shown that the infecting strain was antigenically dissimilar to the strains cultured from the patient's own nose and throat and other strains cultured from the personnel of the operating room (4).

It was thought that such a study of the 54 strains from this epidemic of puerperal fever might indicate whether or not it was a true epidemic coming from a single source and whether or not the organism was being carried by any of the attending staff.

The sources of the 54 strains tested, were as follows: Four were cultured from patients' blood, twenty from patients' vagina, four from metastatic foci, one from the surface of a patient's thigh, one from the axillary abscess of a nurse who pricked her finger with a pin while taking care of one of the fatal cases, one from the peritoneum of a nurse who developed a primary hemolytic streptococcus peritonitis during the course of the epidemic, fourteen from the throats of staff members, seven from the noses of staff members, one from a patient's throat and one from a patient's nose. The two cultures from the patients' nose and throat were obtained in the course of the routine cultures carried out for several months after the epidemic was over and were included because they were the only positive cultures from the noses or throats of the parturient women. Two of the vaginal cultures and one from the patient's thigh were from two patients who did not show the clinical signs of puerperal fever. Three other nose cultures and eight other throat cultures from the staff on repeated transplantation in artificial media lost their hemolytic quality and were discarded.

EXPERIMENTAL.

Five strains from five different puerperal fever cases and one strain from the throat of one of the doctors who had been associated with several of the cases, were first taken for animal inoculation. Three of the patients' strains had been cultured from the blood and two from the vagina. The stock cultures were kept in cooked meat medium in which streptococci may survive for a year or more but they were transferred at intervals of 2 to 3 months. Out of the stock cultures they were transplanted into fresh meat medium for 24 hours and then were grown in phosphate buffered broth for 24 hours further. Rabbits were inoculated 3 days in succession every week with gradually increasing doses of whole culture, first heat killed and then living. A number of animals died of septicemia after the first small dose of living organisms. When the serum showed an agglutinating

titre of 1–2560, the animal was killed and the serum obtained. This generally required 2 months.

Agglutination tests were carried out in the usual way. The serum dilutions were made with phosphate broth and after the organisms were added, the dilutions ranged from 1–80 up to 1–2560. Most of the strains were granular at first but after being transplanted daily for a week or two in phosphate broth they became diffuse. Sterile potato was added to the phosphate broth for the final growth before the agglutination test. The tubes were incubated for 1 hour at 56°C. and the readings made at the end of that time. The reaction was almost always clear-cut and nothing seemed to be gained by keeping the test in the ice box overnight before reading, as advocated by some writers. In our records the degrees of agglutination ranged from ± to 4, while 0 denoted complete absence of agglutination.

TABLE I.

Preliminary Classification of 54 Strains in Main Agglutination Group.

Source	Total	Positive	Negative
Patients' blood	4	2	2
" vagina	20	14	6
" metastatic foci	4	4	0
" thigh	1	1	0
" nose	1	0	1
" throat	1	0	1
Staff, nose	7	3	4
" throat	14	4	10
" abscess	1	1	0
" peritoneum	1	1	0

The technique of absorption of agglutinin was similar to that used in our previous work (4) except that greater accuracy of dosage was attempted. The minimal absorptive dose had to be determined for each serum. This varied from 100 to 150 billion organisms for 1 mil of a 1–20 dilution of serum. After incubating in large flasks, the cultures were centrifuged and washed once in distilled water. They were then suspended in a concentrated form in distilled water and counted by means of a Gates turbidimeter (5). Compared with a known standard, the number of organisms per mil could be determined fairly accurately. Then twice the minimal absorptive dose of organisms was centrifuged and after pouring off the supernatant fluid, 1 mil of a 1–20 dilution of the serum to be tested was added and mixed with the organism sediment. The mixtures were then heated at 56°C. for 1 hour and shaken at quarter-hour intervals. At the end of that time the mixtures were placed in the ice box for the night. Next morning the serum was drawn off and tested for agglutination with the homologous strain, and the absorbing strain as described above.

Results.

A. The Results of the Preliminary Agglutination Tests.—(See Table I.) These preliminary tests seemed to indicate clearly that the great majority of the strains from the patients fell into one agglutinative group. The serum produced by the doctor's throat strain did not agglutinate any of the patients' strains and the organism did not agglutinate in any of the other sera. This seemed to show that the doctor's strain was definitely outside of the main group. Six of the vaginal strains and two of the blood culture strains (J and T) also seemed to be outside of the group for they showed no agglutination in any of the sera. One of these blood culture strains (J) had been used to produce one of the preliminary sera and this serum ran parallel with the other sera but failed to agglutinate the homologous strain. These discrepencies will be discussed below. When two or three strains came from the same patient they always reacted in a similar manner. The strain from the axillary abscess of one nurse and from the peritoneum of the other nurse fell into the main group, three strains from nurses' nose cultures and four from nurses' throat cultures were also included in the main group. These last seven strains came from six nurses, one yielding a positive culture in both nose and throat. At this stage, absorption of agglutinin tests might have been done but vacation intervened and during that time it was decided to produce fresh sera with a fair number of the strains which had been included in the preliminary agglutinative group.

Fourteen cultures were then selected for producing this new series of sera—three from nasal cultures and three from throat cultures of the six nurses and eight strains from the puerperal fever cases. The two blood culture Strains J and T which failed to agglutinate in the preliminary test were used along with six which had fallen into the preliminary agglutinating group. Sera were obtained for these fourteen strains by inoculating rabbits in the same way as before. As these sera were obtained they were tested against all of the patients' strains and all of the other strains which had been included in the preliminary agglutinating group.

B. Results of the Absorption of Agglutinin Tests with the Final Group of Fourteen Sera.—When three of the sera produced by the nurses' strains were absorbed by the patients' strains, it was found that the

TABLE II.

Agglutination and Absorption of Agglutinin Serum H, Produced by Nurse's Nose Strain H.

Tested with Strain H and 7 Patients' Strains.

Serum	Strain	Serum dilutions					
		$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$
Normal.........................	H	0	0	0	0	0	0
"	R	0	0	0	0	0	0
"	L	0	0	0	0	0	0
"	E	0	0	0	0	0	0
"	Ma	0	0	0	0	0	0
"	Mc	0	0	0	0	0	0
"	J	0	0	0	0	0	0
"	T	0	0	0	0	0	0
Serum H unabsorbed....................	H	4	4	4	4	4	3
" " "	R	4	4	4	4	4	3
" " "	L	4	4	4	4	3	3
" " "	E	4	4	4	4	4	2
" " "	Ma	4	4	4	4	3	2
" " "	Mc	4	4	4	4	4	4
" " "	J	0	0	0	0	0	0
" " "	T	0	0	0	0	0	0
Serum H absorbed by Strain H...........	H	0	0	0	0	0	0
" " " " " R............	R	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " L.............	L	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " E.............	E	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " Ma,..........	Ma	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " Mc...........	Mc	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " J.............	J	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0
" " " " " T.............	T	0	0	0	0	0	0
" " " " " "	H	0	0	0	0	0	0

homologous agglutinin could not be completely absorbed by these other strains. On the other hand, when Serum H produced by the strain from another nurse was similarly tested, it was found that all of the patients' strains which were in the preliminary agglutinative group completely absorbed the agglutinin, from this serum. Likewise, when the sera produced by the patients' strains were absorbed by the six different strains from the noses or throats of the six nurses, Strain H, from one nurse's nose, was the only one of the six which would completely absorb the agglutinin from these sera. Of the eight patients' strains used in the last series, five, namely R, L, E, Ma and Mc, produced sera which, with Serum H from the nurse's nose Strain H, gave complete cross agglutination and cross absorption of agglutinin tests. The absorption tests of these six strains with the serum produced by the nurse's strain is shown in Table II. Similar results were obtained with the other five sera produced by the five patients' strains mentioned above. Thus the criteria for the demonstration of the antigenic identity of these six strains were established.

The sera from the two blood culture Strains J and T persistently failed to agglutinate the homologous strains but did agglutinate fully the six identical strains. Furthermore, the two strains J and T producing these sera completely absorbed the agglutinin out of the six identical sera. This will be discussed below. One patient's strain produced a serum which agglutinated the other strains irregularly and this strain did not absorb the agglutinin from the six identical sera. The other two nurses' strains which were included in the preliminary agglutinating group were slow to produce high titred serum and when it was found that these failed to absorb the agglutinin from the six identical sera, they were discarded.

When agglutination and absorption of agglutinin tests were done with the other strains which had fallen into the main agglutinative group in the preliminary tests but against which no immune sera had been prepared, it was found that sixteen more strains completely agglutinated in Serum H produced by the nurses' nose strain and completely absorbed agglutinin in the same absorptive dose as the homologous strain. These are shown in Table III. Six of these strains had come from other foci in four of the patients whose original culture had already been demonstrated to be antigenically identical

with Strain H. Nine of these sixteen strains had come from eight other patients, two of whom had positive vaginal cultures without clinical signs of puerperal fever. One of the sixteen strains came from the axillary abscess of the nurse who had pricked her finger with a pin.

TABLE III.

Final Classification of 54 Strains of Hemolytic Streptococci.

Antigenically identical		Probably identical		Not classifiable	
Complete reciprocal agglutination and agglutinin absorption tests		Complete absorption of agglutinin		No absorption of agglutinin ± agglutination	
Strain	Source	Strain	Source	Strain	Source
L1	Patient's blood	L2	Patient's vagina	Wh	Patient's vagina
Mc1	" "	L3	" peritoneum	Ce	" "
Ma1	" vagina	Mc2	" vagina	Co	" "
R	" "	Mc3	" pleura	Se	" "
E1	" "	Ma2	" abscess	Sc	" "
H	Nurse's nose	E2	" "		And 22 other strains
		J1	" blood		from nose and
		J2	" vagina		throat hospital
		T1	" blood		staff
		T2	" vagina		
		D	" "		
		Ba	" "		
		Wo	" "		
		F	" "		
		P	" "		
		N	" "		
		Bl	" "		
		O'M1	" "		
		O'M2	" thigh		
		M	Nurse's abscess		
		Sm	" peritoneum		

Aside from the sixteen strains, which both by the agglutination and by the agglutinin absorptive tests demonstrated their close antigenic relationship with Strain H, five strains completely absorbed the agglutinin from Serum H, but persistently failed to agglutinate in it. Two of these, Strains J and T, as stated above, came from the blood cultures of two of the fatal cases and two more came from the vaginæ of the same two patients. The fifth came from the peritoneum of

the nurse who developed hemolytic streptococcus peritonitis during the course of the epidemic. In the preliminary tests this strain had agglutinated in the sera produced by the patients' strains.

Five other patients' strains failed either to agglutinate or to absorb agglutinin although one of these, Strain Wh, in the preliminary tests had agglutinated in all of the sera produced by the patients' strains. When this last strain was again tested against these sera it failed both to agglutinate and to absorb agglutinin from them. The results with this strain were irregular both for agglutination and agglutinin absorption. Likewise the serum produced by it gave irregular results. It may have been an incidental strain or have degenerated during the progress of the tests.

DISCUSSION.

It is of interest to note the sources from which the organisms were obtained. Even for those strains which were demonstrated to have the closest antigenic relationships it is evident that the organisms had no specific affinity for any particular organ or tissue and could reside for a time at least in the nasal passage as well as in the vaginal passage without producing clinical symptoms. On the other hand it is evident that the organisms producing the epidemic were in a state of high virulence or invasiveness, for the mortality of those afflicted with the disease was high. Likewise the onset of symptoms was rapid. This was true in the case of the nurse who pricked her finger with a pin and in whom we probably know the exact time of the introduction of the organisms (20 hours between the prick of the finger and the onset of symptoms) as well as in the case of the patients with puerperal fever and the nurse with peritonitis in whom we do not know the exact time of introduction. In rabbits also the organisms proved to be highly invasive (although there would be no reason to expect a parallel susceptibility) for even after a month or more of vaccination, several animals developed septicemia and died following the first small injection of living organisms. This fact would seem to argue against the rationale of specific vaccination during the acute stages or even the chronic stages of the disease itself, as advocated by some. The normal animals could not build up protective substances rapidly against these organisms following intravenous

injection. Whether or not they would have done so, had we injected the organisms by any other route, we cannot tell.

Considering first the results of the preliminary agglutination tests we find that twenty-one out of thirty-one strains cultured from patients, were included in the main agglutinating group. Our chief concern here is to explain the failures if that be possible. Of the ten which failed to agglutinate, one came from the nose and one from the throat of two parturient women without post partum fever after the epidemic was over, being discovered by routine cultures of the nose and throat of all cases admitted for delivery. While it is possible that these patients picked up these organisms after admission into the hospital, it is more likely that they brought them in when they entered and they are evidently not related to the epidemic. Four of the ten strains came from the vaginæ of patients with puerperal fever who were frankly considered to be included in the epidemic. It is possible that these cases were caused by organisms different from the strains responsible for the epidemic and were simply coincident with but not a part of the epidemic. On the other hand, in view of the changes which we know took place in the antigenic and agglutinative characteristics of some of the other strains it is probable that some of these at any rate, failed to agglutinate because they had undergone certain degenerative processes in the course of several months habitation in artificial media. Krumwiede has brought out the fact that these changes do occur with other organisms and it has been observed by others. In such circumstances it is readily understood that although positive results with agglutination and absorption of agglutinin tests are significant of similarity of antigenic properties, negative results do not necessarily prove that similarity did not exist at the time of the first cultivation. It would seem advisable to emphasize this point by repetition namely that positive results mean something but negative results mean nothing, for although strains once positive may become negative, strains originally negative and antigenically different if they change at all, have an infinitely small chance of becoming positive and antigenically identical with another strain.

The other four strains of the ten failures which we are now considering, came from two of the fatal cases (J and T) in the early part of the epidemic, two were vaginal cultures and two were blood cul-

tures. One of these patients (T) was especially cared for by the nurse who pricked her finger with a pin. We have reason to believe that these strains originally were the same as the ones which we have definitely proved to be antigenically identical. The following facts with regard to the behavior of these strains lend weight to this belief. One of these strains, namely J from a blood culture, was used to produce one of the preliminary sera. During the course of inoculation, the serum of the rabbit was tested from time to time and showed gradually increasing agglutinative titre for the homologous strain. When it had attained the desired titre, mentioned above, the animal was killed by bleeding and the serum was taken and stored. About a month's time elapsed before this serum was tested for agglutination with all of the strains and then to our surprise the serum failed to agglutinate the homologous Strain J but ran parallel with the other sera produced by the other patients' strains in that it agglutinated the same strains. Evidently something had happened to Strain J in the meanwhile. On thinking back it was recalled that some difficulty had been encountered in recovering the organism out of the stock culture. It had evidently died off in the supernatant fluid of the cooked meat medium and could only be recovered by transplanting some of the meat. After this, it was noted that the organism was not as clearly hemolytic and grew more diffusely than before. The same thing occurred with several of the strains, notably all four of the strains we are now considering, also the strain from the peritoneum of one nurse and the strain from the axillary abscess of the other nurse as well as two of the four strains from non-fatal cases which, as mentioned above, failed to fall into the main group. Whether this feature of their cultivation, namely their more rapid death in the meat medium than other strains, depended upon some slight variation in the media or in some inherent quality in the strain itself, cannot be determined but the fact that both the blood and the vaginal cultures from two patients should do the same thing, would suggest that the latter explanation is at least a possible one. It is even possible that such changes may have taken place while the organism multiplied within the patient. This cannot be the sole factor however because Strain J did agglutinate in the homologous serum at first and the change came later.

Both of these blood culture Strains J and T, as has been stated above, were included in the group of strains selected for the second series of agglutinative sera. During the course of inoculation these strains were tested for agglutination with their corresponding sera and persistently refused to agglutinate. After continuing the vaccinations for more for 2 months, the sera were tested for agglutination with the six other strains which had by that time demonstrated their antigenic identity. To our surprise, both of these sera fully agglutinated all of these six strains and when these two blood culture Strains J and T were used for absorption, it was found that they both completely absorbed the agglutinin out of the sera produced by the other strains. This is shown in Table II (q. v.). We have, therefore, two strains which are antigenic in the sense that they can produce agglutinating serum. They can also combine with the antibody in serum and carry it down during centrifuging but they persistently refuse to agglutinate. They take part in the first stage of the agglutination process by uniting with the antigen but the second stage, which is the visible one of flocculation and sedimentation, does not take place. We can only be certain of the fact of their union with the antibody by testing the absorbed serum and finding that the agglutinin antibody has been removed. We have, then, two sera for blood culture Strain J, one produced while the organism was agglutinable and one produced after it had lost its agglutinability. So far no differences in agglutinin action have been found in these two sera. The split in the two phases of agglutination as demonstrated by these results when combined with other facts, may throw some light upon the phenomenon as a whole and upon the antigenic characteristics of hemolytic streptococci.

If we turn to Table I to consider the results of the preliminary agglutination tests with the strains cultured from the staff, we find that nine of these were included in the main agglutinative group. Six of these failed to absorb the agglutinin completely from any serum but their own. One (Strain H) from the nose of a nurse was found, by the reciprocal agglutination and absorption of agglutination tests, to be antigenically identical with five of the patients' strains. One (Strain MO) from the axillary abscess of the nurse who pricked her finger, in the later tests lost some of its agglutinability but com-

pletely absorbed agglutinin.   The last (Strain S) from the peritoneum of the nurse with so called primary peritonitis completely lost its agglutinability in the later tests and yet completely absorbed agglutinin from the serum produced by Strain H, one of the six strains proved antigenically identical.   No serum was produced for these last two strains so that we are not able to carry out reciprocal tests but the fact of their power to completely absorb agglutinin makes probable their antigenic identity with the other strains.

When the tests had been completed the question obviously arose whether the nurse, whose nose culture yielded Strain H, which proved to be antigenically identical with five of the patients' strains, was or was not the one who brought the organism into the hospital.   It was found on examining her record that she had not joined the staff until after the epidemic had started.   Whether or not she aided in its continuance cannot be stated.   The fact is demonstrated, however, that the organism could be carried in the nose of a staff member without producing clinical symptoms.

Of the fourteen strains cultured from the staff which failed to fall into the preliminary group, certain ones may have originally been in the epidemic group and have lost their antigenic characteristics between the time of the isolation and the tests.   The changes which we know took place in some of the other strains make it not at all unlikely that this is so.   The original carrier may have been in this group but this can never be determined.

SUMMARY.

1. A study has been made by means of agglutination and absorption of agglutinin tests, of the antigenic relationships of 54 strains of hemolytic streptococci associated with an epidemic of puerperal fever in a large city obstetric hospital.

2. These organisms were cultured from the blood, vaginæ, peritoneal cavities and metastatic foci of patients, from the noses and throats of patients and staff members and from the peritoneum and axillary abscess of two of the nurses.

3. In preliminary agglutination tests twenty-one out of thirty-one cultures from the patients and nine out of twenty-three cultures from the hospital personnel, fell into a single agglutinative group.

4. By reciprocal agglutination and absorption of agglutinin tests, six of these strains were demonstrated to be antigenically identical. Two of these came from patients' blood cultures, three from patients' vaginal cultures and one from the nasal culture of one of the nurses. This nurse was, however, not the original carrier for she did not join the hospital staff until after the epidemic began.

5. By agglutination and complete absorption of agglutinin, sixteen more strains showed their antigenic similarity to, if not identity with, these other six strains. Four of these sixteen came from metastatic foci and two from the vaginæ of four of the five patients which had yielded from another source, the identical strains mentioned in 4. Nine came from eight other patients and one from the axillary abscess of a nurse. No ·reciprocal tests were possible with these sixteen strains because sera had not been prepared against them.

6. Five other strains which completely absorbed agglutinin had evidently lost their ability to agglutinate. Two of these strains were from the blood cultures and two from the vaginal cultures of two fatal cases. One was from the peritoneal cavity of a nurse.

7. Two of these five strains when injected into rabbits in the usual way for the purpose of producing agglutinating antiserum, stimulated the production of sera which agglutinated completely the six antigenically identical strains but failed to agglutinate the homologous strains used to produce the respective sera.

8. These two strains therefore retained the antigenic properties first of stimulating the agglutinating antibody in normal animals and second of uniting with the agglutinating antibody in the serum but lost the ability to bring about the second phase of agglutination —namely flocculation.

9. We have demonstrated, therefore, a more or less permanent splitting of the phenomenon of agglutination.

CONCLUSIONS.

1. A series of twenty-four cases of puerperal fever occurring in the course of 1 month in a certain obstetric hospital constituted a small epidemic apparently caused by a single strain of hemolytic streptococcus of unusual virulence.

2. An organism capable of producing puerperal fever may be carried in the nose of one of the hospital staff without causing clinical symptoms of disease.

3. Careful masking of both nose and mouth should be practiced by all those attendants who may come in contact with parturient women either just before, during or just after labor.

4. Strains of hemolytic streptococci may lose some of their antigenic and agglutinative characteristics if kept on certain artificial media for a period of some months.

5. They may retain their antigenic power both to stimulate agglutinin and combine with agglutinin and at the same time lose their ability to agglutinate.

6. Biological studies employing the agglutination or absorption of agglutinin tests should be carried out as soon as possible after the isolation of the organisms.

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EXPERIMENTAL MEDICINE

SEROLOGICAL DIFFERENTIATION OF STERIC ISOMERS.

By K. LANDSTEINER, M.D., AND J. VAN DER SCHEER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In previous work[1] the conclusion was arrived at that serological specificity depends on the chemical constitution of the reacting groups in general and probably also on their spatial configuration. The latter assumption had been made on account of the reactions observed with antigens containing aromatic compounds substituted in various positions of the benzene nucleus. The experiments dealt with in the present paper were carried out in order to secure conclusive evidence for this conception. Thus we endeavored to determine whether optical isomers can be differentiated by means of serum reactions.

The substances chosen for this purpose were *l-* and *d-*phenyl (para-aminobenzoylamino) acetic acid. These compounds had been used by Ingersoll and Adams[2] in an investigation on the affinity to fibers, of optically isomeric dyes. By diazotization and coupling to dimethylaniline they obtained two isomeric dyes which were absorbed by wool in different amounts; when β-naphtol was used in place of dimethylaniline the dyes were absorbed equally well.

The preparation of the phenyl (para-aminobenzoylamino) acetic acid was carried out in the main according to the directions of Ingersoll and Adams. Phenyl-aminocyanide made by the interaction of benzaldehyde, sodium cyanide, and ammonium chloride in water solution, was hydrolyzed to phenylaminoacetic acid.[3] The *d-l-* phenylaminoacetic acid was resolved into the levo and dextro product by means of fractional crystallization of the *d-*camphor sulfonate and *l-*camphor sulfonate respectively. In the recrystallization of the *l-*amino acid *d-*camphor

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[1] A review of the subject can be found in The chemical aspects of immunity, by H. Gideon Wells, American Chemical Society, Monograph Series, New York, 1925, 77.

[2] Ingersoll, A. W., and Adams, R., *J. Am. Chem. Soc.*, 1922, xliv, 2930; 1925, xlvii, 1169.

[3] *Cf.* Marvel, C. S., and Noyes, W. A., *J. Am. Chem. Soc.*, 1920, xlii, 2264.

sulfonate it was found of advantage to filter off the crystals when the solution had cooled to about 45°. Few recrystallizations were sufficient to obtain a pure product. For the isolation of the *d*-amino acid, inactive camphor sulfonic acid was used. The *d*-amino acid *l*-camphor sulfonate was purified by several recrystallizations.

Both forms of phenylaminoacetic acid were condensed with para-nitrobenzoylchloride to form *l*- and *d*-phenyl (*p*-nitrobenzoylamino) acetic acid $HO_2CC(C_6H_5)$-$HNHCOC_6H_4NO_2$. For recrystallization of this substance thirty times its weight of a boiling mixture of one part of alcohol and two parts of water was used. Crystallization was allowed to take place while stirring. The melting points agreed with those given by the authors named. The nitro compounds were reduced to *l*- and *d*-phenyl (para-aminobenzoylamino) acetic acid by means of ferrous sulfate and ammonia and the final products were recrystallized from water. The levo form had the melting point 168–169°, 0.400 gm. dissolved and made up to 5 cc. with normal HCl gave at 20° a rotation of −14.97 in a 2 dm. tube, with sodium light. For this concentration Ingersoll and Adams give a rotation of −15.00 and the melting point 168–169°. The dextro form had a melting point of 168–169°; 0.400 gm. dissolved and brought up to a volume of 5 cc. with normal HCl at 20° gave a rotation of +14.98 in a 2 dm. tube with sodium light, in accordance with the data of Ingersoll and Adams.

d-l-Phenyl (para-aminobenzoylamino) acetic acid was prepared in the manner described by the authors quoted, from the inactive phenylaminoacetic acid. Melting point 152°.

The levo- and dextro-phenyl (para-aminobenzoylamino) acetic acids will for the sake of convenience be designated as *l*- and *d*-acid. The azoproteins prepared from these amino acids by diazotization and coupling to proteins will be referred to as *l*- and *d*-antigens and likewise the sera obtained by immunization with the azoproteins as *l*- and *d*-immune sera, respectively; *i* denotes the inactive preparations.

Preparation of the l- and d-Antigens for Immunization.—The *l*- and *d*-acids were coupled to proteins in the following manner: 17.12 gm. of the optically active compound were dissolved in 200 cc. water and 25 cc. of 7 normal HCl and diazotized with the required amount of sodium nitrite at a temperature of 0–5°C. with starch iodide paper as indicator; the diazo solution was diluted with ice water to a volume of 500 cc. and poured into a mixture of 500 cc. of horse serum and 500 normal sodium carbonate; coupling was allowed to take place for 10 minutes at 0–5°. By acidification with hydrochloric acid the azoprotein was precipitated and after filtration it was dissolved in a small volume of water by addition of a little normal sodium carbonate. It was reprecipitated from this solution with a large quantity of alcohol and enough hydrochloric acid to flocculate the material. The precipitate was filtered, again treated in the same manner, and the azoprotein was freed from alcohol by redissolving in water and sodium carbonate, and precipitation with hydrochloric acid. It was finally dissolved and brought to a volume of 950 cc. using just enough sodium carbonate to give neutral reaction to litmus

TABLE I.

To 0.2 cc. of the diluted antigen were added 3 capillary drops of *l*-immune serum.

Readings taken after	*l*-Antigen prepared with chicken serum Dilution 1.				*d* Antigen prepared with chicken serum Dilution 1.			
	20	100	500	2500	20	100	500	2500
2 hrs at room temperature	++	+±	+	tr.	+±	0	0	0
Night in ice box	+++	++	++	+±	++	±	f.tr.	0

TABLE II.

To 0 2 cc. of the diluted antigen were added 4 capillary drops of *d*-immune serum.

Readings taken after	*l*-Antigen made with chicken serum Dilution 1				*d* Antigen made with chicken serum Dilution 1			
	20	100	500	2500	20	100	500	2500
2 hrs at room temperature	++	0	0	0	+±	+±	+±	±
Night in ice box	+++	±	0	0	+++	+++	++	+

TABLE III, *a*.

To 0.2 cc. of the diluted antigens were added 3 capillary drops of *l*-immune serum.

Readings taken after:	*l* Antigen made with chicken serum Dilution 1		*d* Antigen made with chicken serum Dilution 1.		*i*-Antigen made with chicken serum Dilution 1.	
	100	500	100	500	100	500
2 hrs at room temperature	+	+	0	0	+	±
Night in ice box	++±	++	±	0	++±	+±

TABLE III, *b*.

To 0 2 cc. of the diluted antigens were added 2 capillary drops of *d*-immune serum.

Readings taken after	*l* Antigen made with chicken serum Dilution 1		*d* Antigen made with chicken serum Dilution 1		*i* Antigen made with chicken serum Dilution 1.	
	100	500	100	500	100	500
2 hrs at room temperature	f tr.	0	+	+	+	±
Night in ice box	±	0	++	++	++	++

and the necessary amount of a salt solution to make the ultimate salt concentration approximately 1 per cent. 50 cc. of a 5 per cent phenol solution was added.

TABLE IV, *a*.

0.2 cc. of *l*-antigen (diluted 1 : 500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated, in 10 cc. To this 3 capillary drops of *l*-immune serum were added. The control tube contains only immune serum and antigen.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	±	+	+	+	±	+	±	+	0	±	f.tr.	+±
Night in ice box	+±	+±	++	++	++	+±	+±	++	tr.	+±	+	++

TABLE IV, *b*.

0.2 cc. of *d*-antigen (diluted 1 : 500) prepared with chicken serum was mixed with 0.05 cc. of a neutral solution containing 1 millimol of the substances indicated in the first line of the table in 10 cc. To this mixture 3 capillary drops of *d*-immune serum were added.

Readings taken after:	1	2	3	4	5	6	7	8	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	+	+	+±	+±	+	+	+	+±	±	0	0	+±
Night in ice box	++	++	++	++	++	++	+±	++±	+±	0	+	++±

TABLE IV, *c*.

The following tests were made like those given in Tables IV,*a* and IV,*b*, but 0.05 cc. of solution containing 0.5 millimol of the *d*-, *l*-, and *i*-acids was added.

Readings taken after:	*l*-Antigen and 3 capillary drops *l*-immune serum, with addition of:				*d*-Antigen and 3 capillary drops *d*-immune serum, with addition of:			
	l-Acid	*d*-Acid	*i*-Acid	Control	*l*-Acid	*d*-Acid	*i*-Acid	Control
3 hrs. at room temperature	0	±	0	+	+	0	±	+±
Night in ice box	±	++	+±	++	++	tr.	+	++±

Immunization.—Two series of seven rabbits each were injected with 15 cc. of the *l*- and *d*-antigens at weekly intervals. In each of the two series one serum of medium strength was produced and in addition some weakly reacting sera.

Antigens for the Tests.—These were prepared in the same way as the antigens for immunization; as protein component chicken serum was used. After coupling, the azoprotein was precipitated with acid, washed with water, and brought into solution by means of sodium carbonate. The quantity of antigen in the solution was determined by precipitation with alcohol and weighing of the dried substance. The dilutions given in the tables are in terms of a 5 per cent stock solution.

The intensity of the reactions is indicated as follows: 0, f.tr. (faint trace), tr. (trace), $\pm$, $+$, $+\pm$, etc.

The immune sera obtained were tested against the *l-*, *d-*, and *i-*antigens with the results given in Tables I, II, and III *a*, *b*.

In addition to the tests presented others were made in order to determine the inhibiting effect on the precipitin reaction,[4] by the *l-*, *d-*, and *i-*acids and other substances, namely; (1) benzoic acid; (2) para-aminobenzoic acid; (3) sulfanilic acid; (4) acetic acid; (5) phenyl-acetic acid; (6) hippuric acid; (7) phenylglycocoll; (8) phenylalanine (Tables IV,*a*, IV,*b*, IV,*c*).

DISCUSSION AND SUMMARY.

The tests presented in Tables I, II, and III show that only in the higher concentration cross reactions do take place, and that there is definite specificity of the two sorts of immune sera for the homologous antigens. Thus it is easy to differentiate the *l-* and *d-*antigens in dilutions 1:100 and upwards. The occurrence of cross reactions can readily be ascribed to the fact that the *l-* and *d-*acids present in the two antigens are identical in every respect but the position of the groups connected with the asymetric carbon atom. The *i-*antigen reacts with both sorts of immune sera as could be expected since it must consist of a mixture of equal parts of *l-* and *d-*antigen.

The reactions of the *i-*antigen appear to be only slightly weaker than those of the homologous ones owing to the fact that the intensity of the reactions diminishes but slowly with increasing dilution of the antigens. It is also to be considered that small differences cannot be judged very accurately.

Tests with two *l-* and four *d-*immune sera[5] not recorded in the tables confirmed the results already discussed.

[4] *Cf.* Landsteiner, K., *Biochem. Z.*, 1920, civ, 280.

[5] Two of these, of medium strength, were obtained from an additional immunization experiment.

Considering that ferments are known to be adapted ordinarily to one type of steric isomers it may be worth noting that antibodies were formed by the same species of animals for optical antipodes.

From the results summarized in Tables IV,*a* and IV,*b* one sees that the *l*- and *d*-immune sera also differentiate clearly between the *l*- and *d*-acids when they are not diazotized and not combined with protein. The *l*-acid inhibits much more the precipitation of the *l*-antigen by the homologous immune serum than the *d*-acid and the converse effect occurs if the inhibiting action is tested on the precipitation of *d*-antigen by *d*-immune serum. The inactive phenyl (para-aminobenzoylamino) acetic acid behaved in such tests as a mixture of *l*- and *d*-acids, *i.e.*, it acted markedly in both cases, more than the heterologous and less than the homologous acid.

The experiments reported bring a definite proof for the view that the steric configuration of antigenic groups is one of the factors determining serological specificity. In the particular case under consideration the mere difference in the position of H and COOH as indicated in the following formulas sufficed to alter the reactivity.

$$
\begin{array}{cc}
NH_2 & NH_2 \\
| & | \\
\bigcirc & \bigcirc \\
| & | \\
CO & CO \\
| & | \\
NH & NH \\
| & | \\
HOOC\!-\!C\!-\!H & H\!-\!C\!-\!COOH \\
| & | \\
\bigcirc & \bigcirc
\end{array}
$$
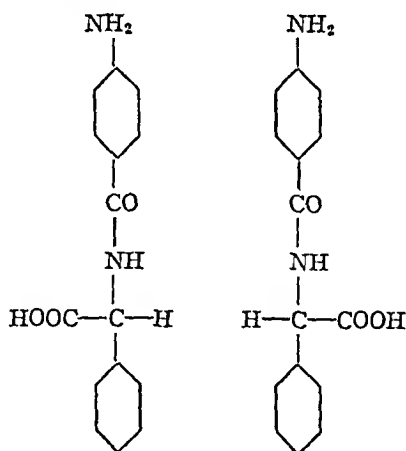
The fact that steric isomers are acted upon selectively by immune sera may be supposed to play a significant part in the serological specificity of carbohydrates such as those discovered in bacterial antigens.

THE INFLUENCE OF CHOLESTEROL ON EXPERIMENTAL TUBERCULOSIS.

By RICHARD E. SHOPE, M.D.

(From the Department of Pharmacology of the State University of Iowa Medical School, Iowa City, Iowa, and the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

It is generally recognized today that the high tuberculosis morbidity and mortality rates among the white races are conjoined with phenomena which indicate that the majority of individuals possess an important, though variable, degree of resistance to the progress of this infection. The nature of this resistance is very obscure. Analogy with more acute diseases suggests the possibility that the mechanisms of acquired immunity as represented by antibody formation and action, phagocytosis, or the still undefined "tissue resistance" may be factors of importance. Such attempts as have been made to determine the place of these factors have led to no decisive results.

The consecutive and rather characteristic series of tissue reactions leading to the formation of the tubercle, the degeneration of the tubercle, and, in favorable cases, to the walling off, fibrosis, or calcification of these abnormal structures and their associated bacilli, suggest as another major possibility that substances chemically less elusive than antibodies, and with a more direct bactericidal action against the tubercle bacillus might be formed or accumulated in the diseased tissue and there act as adjuvants in the healing process. If this were the case, it might be hoped that the recognition of such substances would make possible their artificial quantitative or qualitative alteration with beneficial results.

In reviewing the reported chemical analyses of tuberculous tissues, the most striking variation from the normal is the high value frequently obtained for cholesterol.

Caldwell (1) has conducted the most complete analysis of tuberculous tissues using modern quantitative methods. He found that in normal bovine lymph

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glands cholesterol made up 6.52 per cent of the lipin fraction, or 1.58 per cent of the dry weight of the specimen; while in the walls of tuberculous bovine lymph glands cholesterol made up 13.17 per cent of the fatty substances, or 3.88 per cent of the dry weight. In the caseous material from mesenteric and peribronchial bovine lymph glands cholesterol made up 26.58 per cent of the total lipins, or 5.18 per cent of the dry weight of the specimen. In normal bovine liver he found the cholesterol content of the lipin fraction to be 5.07 per cent, or cholesterol made up 1.6 per cent of the dry weight of the specimen. In the lipins from the walls of liver tubercles cholesterol constituted 14.4 per cent, or 2.25 per cent of the dry specimen. The caseous material from liver tubercles was very rich in cholesterol; the lipin fraction was 26.48 per cent cholesterol, and calculated on the dry weight of the specimen cholesterol made up 4.7 per cent. From these analyses it is evident that cholesterol increases at tuberculous foci from 200 to 300 per cent, depending on the degree of caseation.

In accord with Caldwell's results are those of Jaffé and Levinson (2). These authors found that it was possible to increase further the localized cholesterol by the administration of the substance during the course of the infection. In rabbits which were fed cholesterol in cottonseed oil before and during an infection with bovine tubercle bacilli, the tuberculous lesions showed much more optically active fat than did tuberculous lesions of rabbits that received no cholesterol, indicating that, with an increase in the supply of cholesterol to the animal body, it was possible to increase further the amount of cholesterol or its esters localizing in tuberculous tissues. This they considered due to the fact that the epithelioid cells of the tubercles have their origin from the reticulo-endothelial system which has (as some suppose) to do with cholesterol metabolism. From their experiments, which, so far at least as they concerned the point, were very limited in number, they concluded that cholesterol does not protect rabbits against tuberculosis.

The experiments referred to were of such suggestive importance that they have been repeated in part, in this laboratory, with guinea pigs. The results are in essential agreement with those of previous workers.

The accumulation of cholesterol or cholesterol esters in tuberculous tissues, and especially in the actual anatomical tubercles, suggested the possibility of using this substance in some chemotherapeutic experiments. Since it appeared to be capable of penetrating, and localizing in tubercles, it seemed possible that, if compounds could be made that would contain cholesterol combined with other radicles known to possess bactericidal properties, they might prove to be of therapeutic value. To test the success of such an assumption two series of chemo-

therapeutic experiments have been conducted. In the first series of experiments cholesterol alone has been used in treating guinea pigs experimentally infected with *B. tuberculosis*. In the second series various compounds containing cholesterol or an oxidation product of cholesterol have been used.

<div align="center">EXPERIMENTAL.</div>

I. Chemical.

The cholesterol used was prepared either from human gall stones or hog brains. Cholesteryl chloride was prepared using Rayman's method (3) and cholesteryl anilide and cholesteryl toluide by Walitzky's method (4). Cholesterol sulfuric acid was made according to the method of Mandel and Neuberg (5). A carboxy acid of cholesterol was prepared according to the method of Diels and Abderhalden (6), the last step in their procedure being omitted (refluxing with methyl ethyl ketone). The acid thus obtained had a melting point of 90°C. instead of 290°C. as did the one described by them.

Quinine cholesterylate was prepared by pouring quinine hydrochloride, dissolved in water, into an aqueous solution of sodium cholesterylate until no more precipitate was formed. This precipitate was washed in water and then dried.

The compounds mentioned, with the exception of the two that were water soluble (sodium cholesterylate and sodium cholesterol sulfate), were prepared for injection by dissolving in hot alcohol and pouring the hot alcoholic solution into hot distilled water with constant stirring. A rather stable suspension was obtained. This was heated, to drive off most of the alcohol and to get the suspension down to the strength desired for injection, and then filtered. The sodium salt of the carboxy acid of cholesterol, being water soluble, was used in 2 per cent solution in distilled water except where otherwise specified. Sodium cholesterol sulfate was used in 0.5 per cent solution in distilled water.

II. Biological.

A. Therapeutic Experiments with Cholesterol Alone.

Experiment 1.—(Table I, Chart 1.) 20 guinea pigs were inoculated subcutaneously in the inguinal region with 1 cc. of a very thin suspension of recently isolated human type tubercle bacilli. They were divided into 2 groups of 10 animals each. The infection was allowed to run 3 weeks and then the animals in 1 group were given 4 intraperitoneal injections, each of 4 mg. of cholesterol, suspended in distilled water, at intervals of 3 days. The animals in the control group each received 2 cc. of distilled water intraperitoneally on corresponding days. Animals that obviously died as the result of the injections were discarded. This accounted for 1 from each group. The results are given in Table I.

Experiment 2.—(Table II, Chart 2.) The guinea pigs in this experiment were inoculated subcutaneously with ¼ cc. of a very thin, paper filtered, suspension of recently isolated human type tubercle bacilli. 15 days were allowed to elapse before treatment and then the animals were divided into 3 groups. A group of 7 controls received 7 injections of 2 cc. of distilled water, intraperitoneally, at intervals of 4 days. Another 7 animals received 7 injections, each of 8.8 mg. of choles-

TABLE I.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
33	49	57	66
35	50	58	70
42	53	60	72
50	58	62	112
53	65		
Average........................		50	66

TABLE II.

Days of Survival Following Infection.

Control	Cholesterol	Irradiated cholesterol
63	80	60
64	108	66
71	110	86
75	113	98
95	117	100
96	152	103
96		123
		126
		132
		136
Average: 80	113	103

terol in water suspension, and a group of 11 animals received 7 injections, each of 10 mg. of cholesterol, that had been exposed to the rays from a mercury quartz lamp, after the method of Hess, Weinstock, and Helman (7), on corresponding days. Animals dying as a result of the injections were discarded thus eliminating 1 animal from each of the cholesterol groups. The results are tabulated in Table II.
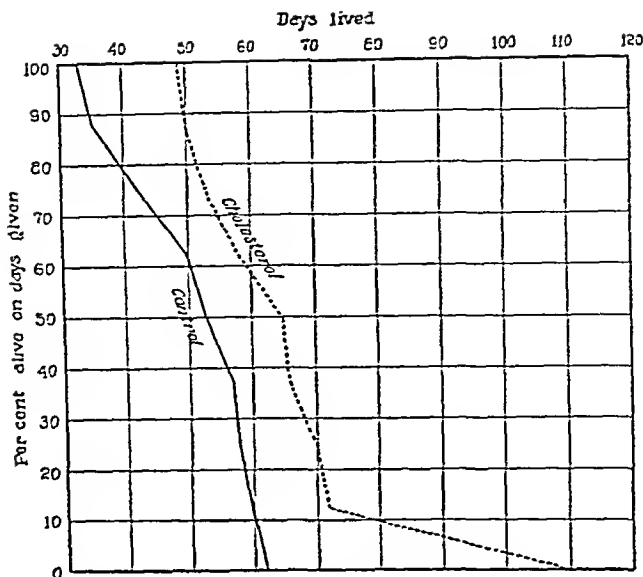
CHART 1. Therapeutic Experiment 1. 1st day. Animals given 1 cc. of a light suspension of freshly isolated tubercle bacilli (human type) subcutaneously. 18th day. Treatment started. 30th day. Treatment stopped. All groups charted.
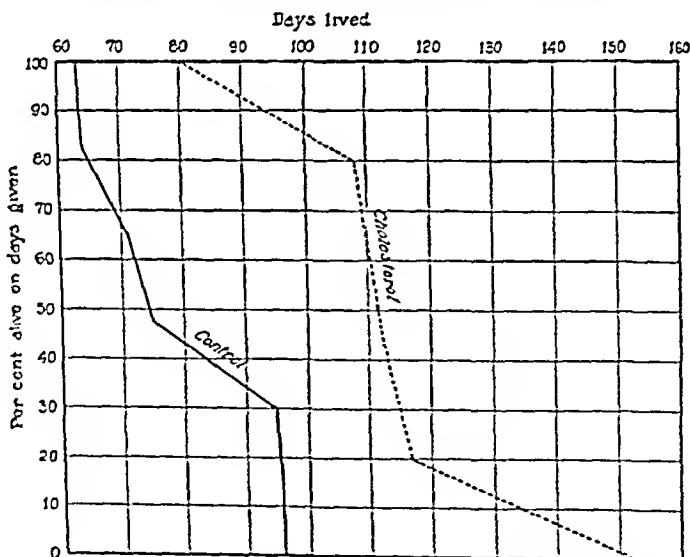
CHART 2. Therapeutic Experiment 2. 1st day. Animals given 0.25 cc. of a paper filtered suspension of tubercle bacilli (human type) subcutaneously. 14th day. Treatment started. 24th day. Treatment stopped. Groups charted: Controls and those receiving cholesterol.

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The work so far reported in this paper was carried out in the Department of Pharmacology at the State University of Iowa Medical School.

The first two experiments had rather consistently indicated a favorable influence of cholesterol on a tuberculous infection in guinea pigs; but the number of animals used was too small to warrant definite conclusions. Only one type of infection had been studied, an acute tuberculosis produced by the tubercle bacillus of human type. It seemed desirable to repeat the experiments with a larger series of animals and a more chronic type of infection.

TABLE III.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
88	130	176	164
94	133	180	166
107	136	182	167
114	138	183	169
121	141	184	169
124	141	184	170
129	146	185	171
143	146	185	174
146	148	192	182
146	149	194	185
153	155	202	190
167	156	205	212
171	160	208	
172	160	239	
175	162		
Average		163	160

Experiment 3.—(Table III, Chart 3.)   60 guinea pigs from Family 13 (inbreds) were injected subcutaneously with 1/10 mg. of Culture H 7156, human type tubercle bacilli.   The culture in this amount was known to produce a chronic type of tuberculosis in guinea pigs.   The infection was allowed to progress untreated for 2 weeks.   Then the animals were divided into 2 groups of 30 each.   1 group of animals received 10 intraperitoneal injections, each of 18 mg. of cholesterol in distilled water suspension, while the animals in the control group received 10 intraperitoneal injections of 3 cc. of distilled water on corresponding days.   The animals were injected on the 14th, 18th, 22nd, 26th, 30th, 34th, 38th, 57th, 68th,
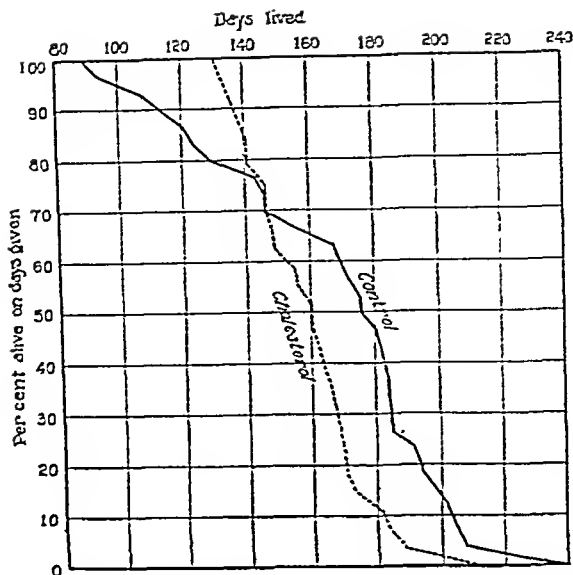
CHART 3. Therapeutic Experiment 3. 1st day. Animals given 1/10 mg. of H 7156 subcutaneously. 14th day. Treatment started. 110th day. Treatment stopped. All groups charted.
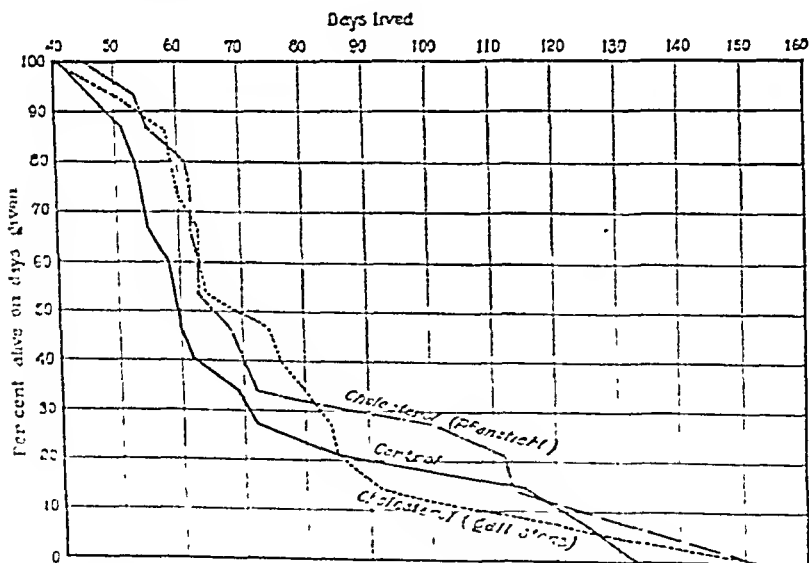
CHART 4. Therapeutic Experiment 4. 1st day. Animals given 1 mg. of H 7156 subcutaneously. 5th day. Treatment started. 125th day. Treatment stopped. All groups charted.

and 110th days following inoculation.   Animals that obviously died as a result of the injections were ruled out of the experiment.   This accounted for 1 of the

TABLE IV.

Days of Survival Following Infection.

Control	Pfanstiehl cholesterol	Gall stone cholesterol
41	45	40
46	53	50
51	55	58
53	61	59
54	62	60
55	62	63
58	63	63
59	63	64
60	68	74
62	70	76
69	72	80
72	100	84
85	112	85
115	113	92
125	152*	121
133	152*	152*
Average: 71	81	76

* Killed on 152nd day to terminate experiment.

TABLE V.

Days of Survival Following Infection.

Control	Cholesterol	Control	Cholesterol
35	28	55	53
42	38	56	55
45	40	58	56
48	42	61	66
49	45	61	68
50	50	64	75
51	50	74	104
53	50	79	
Average..........................		55	54.6

control group, and 3 of the 30 receiving cholesterol.   The results are given in Table III.

Experiment 4.—(Table IV, Chart 4.) Another group of 48 stock guinea pigs, crossbred in heredity, were inoculated subcutaneously in the inguinal region with 1 mg. of Culture H 7156, ten times as large a dose as that previously used, with the intention of getting a relatively more acute infection that would be more comparable to the first two experiments reported in this paper. This time the infection was allowed to run only 5 days before treatment was instituted. Then the animals were divided into 3 groups of 16 each. Group 1 was kept as a control group and received 10 intraperitoneal injections of 3 cc. of sterile distilled water on the 5th, 8th, 12th, 19th, 23rd, 27th, 31st, 103rd, 111th, and 121st days following infection. Group 2 animals received 10 intraperitoneal injections, each of 21 mg.
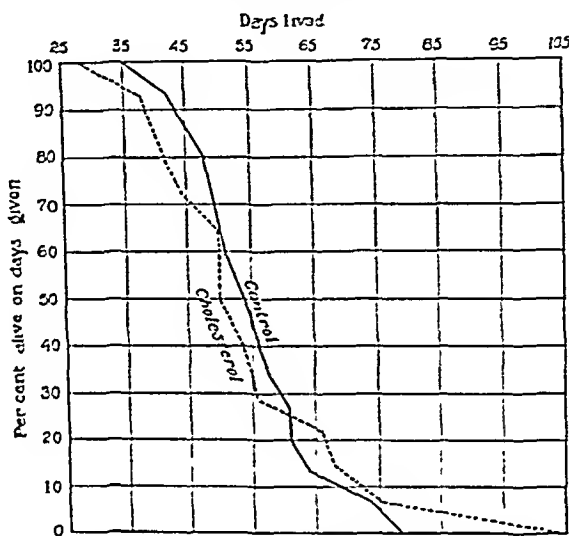
CHART 5. Therapeutic Experiment 5. 1st day. Animals given 1/5 mg. of Bovine 14 subcutaneously. 3rd day. Treatment started. 16th day. Treatment stopped. All groups charted.

of Pfanstiehl cholesterol (hog brain) in watery suspension, on corresponding days; and Group 3 animals received intraperitoneal injections of 21 mg. of cholesterol, prepared from human gall stones, on corresponding days. The results are given in Table IV.

Experiment 5.—(Table V, Chart 5.) 32 guinea pigs were inoculated subcutaneously with 1/5 mg. of a culture of Bovine 14. The infection was allowed to progress 3 days and then the animals were divided into 2 groups of 16 each and treatment was begun. The control group animals each received 5 intraperitoneal injections of 3 cc. of distilled water on the 3rd, 6th, 10th, 14th, and 19th days following infection. The second group each received 5 intraperitoneal injections,

each of 21 mg. of cholesterol, on corresponding days. 1 of the animals receiving cholesterol died of a peritonitis and was discarded. The results are given in Table V.

B. *Therapeutic Experiments with Compounds Containing Cholesterol or Derivatives of Cholesterol.*

Experiment 6.—(Table VI.)   The guinea pigs in this experiment received 1 cc. of a very thin suspension of recently isolated human type tubercle bacilli subcutaneously.   They were then divided into groups of 10 each and caged by groups. 3 weeks following inoculation treatment was begun.   1 group received 4 intraperitoneal injections, each of 3 mg. cholesteryl chloride at intervals of 3 days. The second group received 2 mg. cholesteryl anilide, the third group 1.2 mg.

TABLE VI.

Days of Survival Following Infection.

Control	Cholesteryl chloride	Cholesteryl toluide	Cholesteryl anilide
33	32	32	33
35	49	37	41
42	50	53	46
50	62	55	53
53	64	56	54
57	65	66	58
58	66	87	58
60	80	95	60
62	98		60
			85
Average: 50	63	60	55

cholesteryl toluide, and the controls 2 cc. of distilled water on corresponding days. Individuals dying during the period of the injections were discarded. The results are given in Table VI.

Experiment 7.—(Table VII.)   The guinea pigs in this experiment received ¼ cc. of a very thin, paper filtered suspension of recently isolated human type bacilli subcutaneously.   They were then divided into 2 groups of 7 each and 15 days were allowed to elapse before treatment was begun.   Then the animals in 1 group were given 7 intraperitoneal injections, each of 10 mg. of sodium cholesterol sulfate, at intervals of 4 days.   The animals in the control group received 2 cc. intraperitoneal injections of distilled water on corresponding days. The results are given in Table VII.

Experiment 8.—(Table VIII.)   60 guinea pigs from Family 13 (inbreds) were injected subcutaneously with 1/10 mg. of Culture H 7156, human type of tubercle

bacilli, and divided into 2 groups of 30 animals each. The infection was allowed to progress untreated for 2 weeks. Then the animals in 1 group were given 10 intraperitoneal injections, each of 9 mg. of quinine cholesterylate in distilled water suspension, on the 14th, 18th, 22nd, 26th, 30th, 34th, 38th, 57th, 68th, and 110th

TABLE VII.

Days of Survival Following Infection.

Control	Sodium cholesterol sulfate	Control	Sodium cholesterol sulfate
63	61	95	99
64	71	96	111
71	77	96	126
75	97		
Average...........................		80	92

TABLE VIII.

Days of Survival Following Infection.

Control	Quinine cholesterylate	Control	Quinine cholesterylate
88	105	176	170
94	122	180	171
107	132	182	176
114	133	183	177
121	151	184	184
124	152	184	185
129	158	185	186
143	159	185	190
146	160	192	254
146	161	194	256*
153	162	202	
167	165	205	
171	166	208	
172	168	239	
175	169		
Average...........................		165	168

* Killed on 256th day to terminate experiment.

days following inoculation. The animals in the control group received 3 cc. injections of distilled water intraperitoneally on corresponding days. Animals dying obviously as a result of the injections were discarded. The results obtained are given in Table VIII.

Experiment 9.—(Table IX.) This experiment was made up of 7 groups containing 15 guinea pigs each, crossbred in heredity. The organism used in producing the infection was H 37, a human type tubercle bacillus. The animals were inoculated subcutaneously with 1/5 mg. doses. The treatment administered to each group was as follows: 1 group received intraperitoneal injections of 0.1 mg. sodium cholesterylate twice weekly throughout the experiment and commencing 1 week before the time of inoculation. The other 4 groups of treated animals received sodium cholesterylate intraperitoneally on corresponding days but in the following doses: 0.5 mg., 1 mg., 5 mg., and 10 mg. The remaining 2 of the 7

TABLE IX.

Days of Survival Following Infection.

Controls Distilled H₂O	Controls No injections	0.1 mg. sodium cholesterylate	0.5 mg. sodium cholesterylate	1 mg. sodium cholesterylate	5 mg. sodium cholesterylate	10 mg. sodium cholesterylate
49	97	61	67	116	51	60
82	106	80	131	138	51	68
89	114	80	132	140	82	68
96	122	101	137	142	89	70
96	123	103	157	143	97	72
98	132	107	157	145	98	129
101	158	110	166	262	112	164
101	224	167	167	266	162	212
130	236	168	170	266	167	235
146	255	182	175	278	183	
174	258	184	184	326	184	
228	265	286	321	342	196	
244	321	346*	346*	346*	346*	
255	346*	346*	346*	346*	346*	
265	346*			346*		
Average: 143.6	206.9	165.8	189.7	240.1	154.5	119.7

* Killed on 346th day to terminate experiment.

groups of guinea pigs were maintained as controls. 1 of these control groups received intraperitoneal injections of 1 cc. of distilled water on days corresponding to the injections of sodium cholesterylate. The other group received nothing, in the way of injections, after they had been inoculated. All were maintained on a diet of 30 gm. of cabbage per animal per day with hay, oats, and water *ad lib.* Animals obviously dying as the result of the injections were discarded. This eliminated 1 animal apiece from the groups receiving 0.1, 0.5, and 5 mg. sodium cholesterylate. 10 mg. of sodium cholesterylate was obviously an overdose and 6 of the 15 animals in that group died as a result of the injections. The other 9 in

that group then had the apparent toxicity of the large overdose of sodium cholesterylate to deal with in addition to their tuberculosis. The results of this experiment are given in Table IX.

Summary of Results Obtained in Therapeutic Experiments.
A. Results with Cholesterol.

In Experiments 1 and 2, cholesterol definitely prolonged the lives of the animals receiving it. When the significance of these results (8) was determined mathematically it was found that, in Experiment 1, $P = 0.05$.** In Experiment 2 the value of P for the group receiving cholesterol was 0.01 and for the group receiving irradiated cholesterol was 0.07. In these two experiments the infection was of an acute type, the culture very virulent, and the number of infecting organisms relatively small.

In Experiment 3, contrary to what might perhaps have been expected of a chronic infection, cholesterol proved ineffective, the value for P being 0.65. The less resistant animals in the experiment perhaps responded to the treatment with cholesterol, since, in comparing the two series of animals in Table III, it will be noted that among the first animals to die in each series those treated with cholesterol quite definitely outlived the control animals dying in corresponding order.

In Experiment 4 the animals receiving cholesterol tended to outlive the control animals. The margin of advantage shown in Table IV, however, is not mathematically significant since for the group receiving Pfanstichl cholesterol (hog brain) $P = 0.35$, while for the group receiving gall stone cholesterol $P = 0.60$. Clearly then, the apparent advantage is of no significance. The infection in this experiment proved to be more rapid than in the preceding one, but it was still not the same type of acute infection as in the first two experiments. Whereas, in the first two experiments, the infection was one with a

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** In calculating the significance of the results obtained, P is a measure of probability. A value of $P = 0.03$ indicates that the results are significant mathematically and means that the chances are 33 to 1 that the experiment can be repeated with a similar outcome. Values above 0.03 are considered to indicate that the results are not mathematically significant. Values for P below 0.03 are considered very significant as, for instance, $P = 0.01$ where the chances of being able to repeat the experiment are 100 to 1.

very small dose of very virulent organisms, here we had an overwhelming infection with only a moderately virulent culture. Experiment 4 demonstrated, as would be expected, that there is very little difference in the action of cholesterol from different sources.

In Experiment 5, in which a bovine type of infection was used, cholesterol was ineffective, P being 0.9.

B. Results with Compounds Containing Cholesterol or Derivatives of Cholesterol.

In Experiment 6, while cholesteryl chloride, toluide, and anilide gave some apparent prolongation of the lives of the guinea pigs treated, the results do not prove to be mathematically significant since the P for cholesteryl chloride is 0.1, for cholesteryl toluide 0.25, and for cholesteryl anilide 0.41. The degree of action in each group was roughly proportional to the amount of cholesterol received with each compound.

In Experiment 7 sodium cholesterol sulfate did not significantly prolong the lives of the animals treated, P in this case being 0.28.

In Experiment 8 quinine cholesterylate was ineffective in prolonging the lives of guinea pigs treated with it. P in this case was 0.65.

In Experiment 9 sodium cholesterylate, in doses ranging from 0.1 mg. to 10 mg., was used in treating guinea pigs experimentally infected with *B. tuberculosis*. Scattered experiments on small numbers of animals had previously given some indication that sodium cholesterylate exerted a favorable influence on tuberculous guinea pigs, several animals outliving untreated animals by long periods of time and showing very little tuberculosis when they were killed at the termination of the experiments. The range of doses used was chosen in the hope of finding an optimum at which the compound was most effective. Two groups of untreated guinea pigs were included. One of these, the true control group, received the same amount of handling and incidental trauma from intraperitoneal injections of distilled water as did the treated animals. The other group of untreated animals was included to obtain some data concerning the actual effect on tuberculous guinea pigs of repeated handling and trauma of injection. They were undisturbed except for weighing and cage cleaning after the time of inoculation. As might perhaps have been expected they outlived

the true control group. Their advantage in days lived following inoculation was significant as P was equal to 0.03. The animals receiving 0.1 mg. sodium cholesterylate showed a somewhat greater average length of life than did the true control group but the difference was not significant as P equaled 0.5. The animals receiving 0.5 mg. sodium cholesterylate showed a still greater prolongation of life than the controls but it was not of significance mathematically as P equaled 0.15. The animals receiving sodium cholesterylate in 1 mg. doses showed an average length of life greater than any of the other groups. The prolongation of life in this group was very significant mathematically as P equaled less than 0.01. The two larger doses of sodium cholesterylate used were obviously too toxic and the animals in these groups had the toxicity of the therapeutic agent to deal with in addition to their tuberculous infection. For the group receiving 5 mg. injections of sodium cholesterylate P equaled 0.82, and for the group receiving 10 mg. injections no calculation was made.

The group of animals receiving 1 mg. injections of sodium cholesterylate appeared to outlive the group of animals that had received no injections or were not handled. The difference, however, was not mathematically significant as P in this case equaled 0.35.

I am very grateful to Dr. John W. Gowen for having calculated the mathematical significance of the results obtained in these experiments. Without his concise evaluation of the data obtained accurate interpretation or analysis of the findings would have been impossible.

DISCUSSION.

Four types of experimental tuberculosis in guinea pigs have been treated with cholesterol administered intraperitoneally. In two experiments, in which an acute type of tuberculosis, due to a relatively small number of very virulent organisms, was treated, the animals receiving cholesterol were definitely benefited. They outlived their untreated control animals by a mathematically significant margin. Cholesterol, however, failed to prolong significantly the lives of guinea pigs exhibiting the other three types of tuberculosis treated. It did not benefit animals having a chronic type of tuberculosis produced by inoculation with a relatively small dose of organisms of low virulence, or those having a more acute type of tuberculosis produced by inocula-

tion with a large dose of organisms of low virulence. It did not prolong the lives of animals infected with a quite virulent strain of bovine type tubercle bacilli.

No explanation of the mode of action of intraperitoneally administered cholesterol can be offered as a result of the work nor is any explanation apparent for the differing results with different types of tuberculous infection. The course of the experiments, and particularly the results of the postmortem examinations give no ground for assuming any direct bactericidal action for cholesterol or any direct and pronounced effect in promoting the healing processes. The connective tissue present in and about the lesions was no greater in treated than in untreated animals, and scars or calcified areas or other evidences of healing processes were no more in evidence. The lymph nodes showed no tendency to regressive changes. Lesions of the usual character prevailed and finally caused the death of most of the animals.

The results with compounds containing cholesterol or with derivatives of cholesterol were, as a whole, quite unpromising. Cholesteryl chloride, cholesteryl toluide, cholesteryl anilide, sodium cholesterol sulfate, and quinine cholesterylate did not benefit tuberculous guinea pigs to a significant degree. Sodium cholesterylate, however, in certain doses proved to be quite effective. 1 mg. given twice weekly very significantly prolonged the lives of the animals receiving it whereas smaller or larger amounts that were tried were less effective. In the case of sodium cholesterylate in optimum dosage, the duration of life was little greater in the animals receiving it than in animals bearing a similar infection but not subjected to any handling or injection trauma. However, a comparison of this sort is unfair for, although sodium cholesterylate failed to do much more than "nothing at all" it very markedly prolonged the lives of animals receiving it when compared to control animals undergoing precisely the same inevitable trauma and handling. The mode of action of sodium cholesterylate is equally as obscure as that of cholesterol.

It is conceivable that the results attained with sodium cholesterylate in optimal dosage may give a starting point for the production of other compounds more effective in the treatment of tuberculosis. Experiments with this view in mind are in progress at the present time.

SUMMARY.

1. Cholesterol, administered intraperitoneally, in these experiments definitely prolonged the lives of tuberculous guinea pigs when the infection was of an acute type produced by inoculation with a small dose of very virulent human type organisms.

2. Intraperitoneally administered cholesterol did not definitely prolong the lives of tuberculous guinea pigs when the infection was of the chronic type produced by the injection of a small dose of human type tubercle bacilli of relatively low virulence, or when the infection was more acute owing to the injection of a large dose of organisms of low virulence. It had no beneficial effect on an acute type of infection produced by the bovine type organism.

3. Cholesteryl chloride, cholesteryl toluide, cholesteryl anilide, sodium cholesterol sulfate, and quinine cholesterylate did not significantly prolong the lives of tuberculous guinea pigs.

4. Sodium cholesterylate, in optimal dosage, definitely prolonged the lives of tuberculous guinea pigs.

5. There was a significant shortening in the duration of life of tuberculous guinea pigs subjected to the trauma of intraperitoneal injection and repeated handling as compared with tuberculous guinea pigs that were not handled or traumatized by intraperitoneal injections.

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CHEMICAL CHANGES IN THE BLOOD OF THE DOG IN EXPERIMENTAL PERITONITIS.

By THOMAS G. ORR, M.D., AND RUSSELL L. HADEN, M.D.

(From the University of Kansas School of Medicine, Kansas City, Kansas.)

In previous publications, we have recorded the chemical changes in the blood occurring in pyloric and intestinal obstruction (1, 2). Since distension of the intestine and paralytic ileus are often associated with general peritonitis, a study of the blood chemistry has been made for comparison. It has been suggested that the cause of death in general peritonitis may be due to an intoxication from the intestine rather than to the peritoneal infection.

Method.

Dogs were used for the experimental studies. All operations were done under ether anesthesia with aseptic technique. Peritonitis was produced by ligating the appendix with tape. The animals were kept in a warm room in metabolism cages and allowed to drink water *ad libitum*. Blood for chemical analysis was drawn from the jugular vein daily and at times twice daily if the animal appeared quite ill.

The non-protein nitrogen was determined by the method of Folin and Wu (3), the urea nitrogen by the Van Slyke and Cullen (4) modification of the Marshall method and the carbon dioxide-combining power by the method of Van Slyke (5). The chlorides were determined on the tungstic acid filtrate as suggested by Gettler (6).

OBSERVATION.

Normal dogs weighing from 8 to 13 kilos were used. In all cases the appendix was snugly ligated with tape completely blocking the blood supply. General peritonitis was produced in this way in less than 50 per cent of the animals.

In those dogs developing general peritonitis early changes in the blood chemistry were usually noted. There was a fall in the blood chlorides and a rise in the urea and non-protein nitrogen. The rise in

TABLE I.

Blood Findings in Experimental General Peritonitis.

Dog No.	Day after operation	Blood			CO₂-combining power
		Amount per 100 cc.			
		Total non-protein nitrogen	Urea nitrogen	Chlorides	
		ms.	*ms.*	*ms.*	*vol. per cent*
1	0	26.8	16.8	450	43.8
	1	30.6	10.6	400	45.7
	2	91.5	46.8	330	28.7
2	0	25.4	11.2	490	28.7
	1	42.2	11.9	430	34.3
	2	32.6	21.0	410	34.3
	3	52.0	27.3	380	36.2
	4	31.6	23.1	370	45.7
	5	34.9	13.3	420	43.8
	6	27.3	14.0	380	41.9
	7	22.8	11.9	450	43.8
	8	37.5	20.3	450	49.0
	9	34.5	16.8	430	46.6
	10	50.8	26.6	420	43.8
	11	78.0	45.5	400	32.4
	12	258.0	123.3	340	28.7
3	0	27.3	15.4	560	38.1
	1	27.0	18.2	510	38.1
	2	32.3	20.3	470	38.1
	3	71.4	52.5	440	45.7
4	0	28.0	15.4	460	38.1
	1	40.0	18.9	410	40.0
	2	35.3	21.7	360	38.1
5	0	25.2	13.31	480	34.3
	1	24.2	12.61	460	38.1
	2	28.2	16.81	400	40.0
	3	23.5	11.91	400	36.2
	4	20.2	9.81	400	38.1
	5	56.3	31.51	400	27.5
6	0	32.3	13.3	470	34.3
	1	33.0	12.6	410	45.7

TABLE I—*Concluded.*

Dog No.	Day after operation	Blood			CO₂-combining power
		Amount per 100 cc.			
		Total non-protein nitrogen	Urea nitrogen	Chlorides	
		mg.	*mg.*	*mg.*	*vol. per cent*
6	2	47.2	17.5	370	43.8
	3	31.2	15.4	360	52.0
	4	31.8	16.8	380	41.9
	5 a.m.	34.5	17.5	350	14.3
	5 p.m.	45.8	22.4	320	10.5
7	0	28.0	10.2	450	40.0
	1	31.9	14.0	390	40.0
	2	—	51.3	320	—
8	0	23.4	8.4	490	35.3
	1	22.1	8.4	480	29.6
	2 a.m.	53.2	36.8	440	22.1
	2 p.m.	89 8	49.0	370	—

the nitrogenous elements usually started from 1 to 3 days before the death of the animal and increased until death. The carbon dioxide-combining power did not show any constant change. In some instances there was little or no change, in others a slight increase and in still others a decrease (Table I).

In all animals included in this report a well developed general peritonitis throughout the entire abdominal cavity was found at autopsy. The animals lived from 2 to 12 days following the operation with an average length of life of $4\frac{1}{3}$ days.

DISCUSSION.

The changes here noted in the blood chlorides, urea nitrogen and non-protein nitrogen resemble those observed in pyloric and high intestinal obstructions. In those two conditions an alkalosis develops which is not observed in general peritonitis.

Since the clinical manifestations of acute high intestinal obstruction and general peritonitis are strikingly alike and the chemical changes in

the blood are similar, it seems quite probable that the cause of death may be somewhat similar.

CONCLUSION.

1. A study of the blood chlorides, urea and non-protein nitrogen and the carbon dioxide-combining power in experimental general peritonitis is here reported.

2. The similarity between the chemical changes in high intestinal obstruction and general peritonitis is noted. These chemical changes suggest that the cause of death may be, at least in part, the same in the two diseases.

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THE EFFECT OF OXIDATION OF FILTRATES OF A CHICKEN SARCOMA (CHICKEN TUMOR I—ROUS).

By J. HOWARD MUELLER, Ph.D.

(*From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston.*)

In the course of experiments with the Rous chicken sarcoma, results were obtained which suggested that infectivity of the filtrates was rather quickly destroyed by oxidation. Since the time of Rous' original work on the tumor it has been recognized that saline filtrates become inactive relatively soon at incubator temperatures, and the similar phenomenon in "primary cultures" of the tumor was ascribed by Gye (1) to oxidation in his first paper. Since that time, however, due in part to the work of Baker (2) on the effect of trypsin in Rous sarcoma filtrates, Gye has inclined to the belief that this "auto-inactivation" in saline at 37° was due to proteolytic ferments from the tissue acting on a non-living protein specific factor. He has shown (3) that it may be considerably delayed by HCN in small amounts, which he supposes to be due to a poisoning of the enzyme. Hydrocyanic acid in low dilutions, however, is stated by Oppenheimer (4) to have little deleterious influence on proteolytic enzymes, whereas from the work of Warburg and others it is known to inhibit certain oxidative phenomena almost specifically.

A preliminary experiment showed such striking results when free oxygen was excluded from candle filtrates of the tumor that it seemed worth while to study the phenomenon more carefully. There seemed to be some reason to hope that a partial explanation might be offered for the irregular and discordant results obtained by ourselves and other workers in attempts to repeat the experiments described by Gye (1) in support of his theory of tumor etiology. If, for example, it were possible to show that, like certain bacterial enzymes and toxins (5) an oxidative destruction could be reversed by reducing

agents, the relationship of the phenomenon to Gye's experiments would be evident.

<div align="center">EXPERIMENTAL.</div>

Filtrates of healthy, rapidly growing Rous sarcomas were prepared by grinding the tissue with coarse carborundum in a mortar and suspending the ground mass in saline. The suspensions were centrifuged half an hour, filtered through a thin layer of paper pulp, and finally through a Mandler filter. Such filtrates were perfectly clear, and varied from yellow to pink in color depending on the amount of hemorrhage in the tumor.

Experiment I.

A filtrate prepared as described was divided into two parts. To one was added a solution of cysteine (pH 7.6) to give a final concentration of 1–2000, and after mixing it was covered with melted vaseline (6). A trace of methylene blue added to a similarly prepared test lot was decolorized in 3 or 4 minutes, and remained so for several days at 37°. Nothing was added to the second tube, nor was it sealed by vaseline. Both were incubated at 37°, and small portions removed at intervals for inoculation.

		Chick 1	Chick 2
1. Cysteine Mandler filtrate	2½ hrs. 37°	++++	+++
2. Plain " "		+++	++
3. Cysteine " "	9½ " 37°	+++	++
4. Plain " "		++	±
5. Cysteine " "	23 " 37°	+++	+
6. Plain " "		−	−

Experiment II.

The experiment was repeated and amplified by including a parallel test of HCN in a dilution of about 1–10,000.

Experiment II.

	1 hr.		2 hrs.		3 hrs.		5 hrs.		22 hrs.	
Chick....	3	4	5	6	7	8	9	10	11	12
1. Plain filtrate..............	++	+++	++	+	+	−	+	++	−	−
2. Filtrate + HCN...........	++	−	+	−	++	+++	++	++	−	−
3. " + cysteine.... ...	+++	+++	+++	++	+++	+++	+++	++++	++++	++++
4. " + HCN + cysteine	+++	+++	+++	+	+++	++++	+++	++++	+++	−

Experiment III.

This experiment was designed to compare the action of serum with that of anaerobic conditions induced by cysteine, and to test, at the same time, the effect of simple sealing with vaseline, and of an inert protein such as gelatin. Twelve tubes were prepared, additions of rabbit serum, both fresh and heated to 56° for 20 minutes, saline, of 5.4 cc. of a Mandler filtrate, and 3.0 per cent gelatin, and 0.05 per cent cysteine were made as indicated below. They were then incubated at 37° and portions removed at intervals for testing. Injections of 0.5 cc. were made in each case.

Tube / Chick	8 hrs.		24 hrs.								70 hrs.		7 days			
	13	14	15	16	17	18	19	20	21	22	23	21	25	26	27	28
1. Fresh rabbit serum 0.6 cc..............	+	+			—	—							—			
2. " " " sealed with vaseline	~	+			—	—				†			—			
3. Fresh rabbit serum 0.6 cc. + cysteine, sealed....	+-+-+-+	+-+-+-+			+-+-+-+	+-+-+-+							—	—	—	
4. Heated rabbit serum 0.6 cc..........	~	⧺														
5. " " " " sealed.....	+	+														
6. " " " + cysteine, sealed....	+-+	+-+-+			+-+-+-+	+-+-+-+							—	—	—	
7. Saline 0.6 cc...........										††						
8. " " " sealed...........			—	—			—	—			—	—	—	—	—	
9. " " " + cysteine, sealed.......			+-+-+-+	+-+-+-+			+-+-+	+-+-+			+-+	+-+-+-+				
10. 3% gelatin 0.6 cc...........			—	—			—	—			—	—	—	—	—	
11. " " " sealed...........			—	—			—	—			—	—				
12. " " " + cysteine, sealed...			+-+-+-+	+-+-+-+			+-+-+-+	+-+-+			+-+-+-+	+-+-+-+				

† Both died in 9 days without tumors, and with no evident reason.

While there are some irregularities in the second experiment it is quite clear that (1) a few hours incubation at 37° will render Mandler filtrates of this tumor non-infectious, (2) that a concentration of 1–2000 of cysteine and protection from the air will defer this loss of infectivity for more than 24 hours and occasionally, at least, much longer, and (3) that cysteine is more effective than a dilution of 1–10,000 HCN, which, as a matter of fact, appears to have a slight deleterious action. Other experiments have shown that HCN in this concentration will regularly preserve infectivity of filtrates for some hours, but we have not seen it persist for as long as 24 hours in any experiment.

The possibility now suggested itself that the extreme variability of infectiousness of candle filtrates of this tumor might be due in part to oxidative changes. Great annoyance is caused by these irregularities which may result in completely inactive filtrates over a considerable period of time, and more frequently in isolated cases. The following experiments were carried out.

Experiment IV.

Tissue from a rapidly growing tumor was minced finely and two lots of 4 cc. each ground in glass mortars with carborundum. One lot was then suspended in 200 cc. of a freshly prepared solution of cysteine hydrochloride 1–2000 in saline brought to pH 7.4 with NaOH. Both portions were centrifuged 15 minutes, the cysteine lot after covering with vaseline. Each lot was then filtered through a layer of paper pulp about 5 mm. thick, and then through Mandler candles. The portion containing cysteine was protected as far as possible from the air by vaseline during both filtrations. Although the paper pulp filtrate still contained enough unoxidized cysteine to decolorize methylene blue, the Mandler filtrate did not. That is to say, protection of the solution containing cysteine had been by no means thorough during the two filtrations. The reagent was gradually oxidized, and by the time the candle filtrate was obtained, it had been completely changed. Probably, however, it had spared to a considerable extent the oxidation of other components of the filtrate. The two Mandler filtrates were then tested.

		Chick 29	Chick 30
Plain	Mandler filtrate	+	+
Cysteine	" "	++++	++++

The filtrate in which oxidation had been partially prevented was strikingly more potent than that made in the usual way. Tumors

began to appear on the 8th day, grew rapidly, and killed in both cases on the 22nd day, while the control tumors appeared only after 14 and 20 days respectively, and were both small.

Experiment V.

The same type of experiment was repeated in such a way as to accentuate the differences due to oxidation. Tumor tissue was finely minced and 2 cc. transferred to each of two large test-tubes. A few glass beads were added to each tube and 50 cc. of saline. One tube was stoppered so as to include about 20 cc. air, while to the second, a fresh solution of cysteine, pH 7.4, was added to a final concentration of 1–2000, then completely filled with melted vaseline, and a stopper inserted in such a way that no air bubbles remained. After the vaseline had solidified, both tubes were shaken for $\frac{1}{2}$ hour, fresh air being admitted to the first tube three times. The saline suspensions were then centrifuged, and filtered through Mandler filters without previous paper pulp filtration. The cysteine lot was protected throughout from the air by liquid paraffin, and a test lot of the Mandler filtrate decolorized methylene blue promptly, showing that unoxidized cysteine remained.

		cc.	Chick 31	Chick 32
Cysteine filtrate,		0.5	++++	+++
"	"	0.25	++++	+++
"	"	0.1	++++	+++
Plain	"	0.5	—	—
"	"	0.25	—	—
"	"	0.1	—	—

In this experiment one cannot be certain to what extent the mechanical effect of shaking may have played a part, since this was obviously greater in the tube containing air. The two experiments taken together indicate clearly that a loss in potency takes place during the preparation of filtrates of this tumor which is due to oxidation and may be largely prevented by adding cysteine and protecting from the air. It is therefore probable that variations in potency of filtrates made in the ordinary way are due, at least in part, to this factor.

Without considerable experience with this method it would be hazardous to assume that active filtrates may be obtained invariably by such means, and obviously in carrying out experiments of many kinds it is highly desirable to know that active filtrates are being used. Filtrates of this tumor which become inert within a few hours at 37°

3. The variability of infectiousness of candle filtrates is due in part at least to oxidative changes which take place during their preparation; and more active extracts may be obtained by preventing these by means of cysteine.

4. For elaborate experiments it is possible to preserve filtrates in the ice box with cysteine and vaseline until test inoculations have established their infectivity.

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THE RELATION OF THE CAPSULAR SUBSTANCE OF B. COLI TO ANTIBODY PRODUCTION.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

In several previous communications[1] an early mutation of certain capsulated strains of *B. coli* was described. The strains were obtained from the ileum of young calves, dying as a result of the multiplication of *B. coli* in the small intestine and the resulting general intoxication or septicemia. The original colony on agar plates sent out lateral expansions or wings much thinner than the original nucleus of the colony. The bacteria making up this secondary growth were without a capsule and about 1/20 to 1/25 the virulence of the nucleus when injected into the peritoneal cavity of guinea pigs. The bacteria of the colony nucleus were agglutinated only in very low dilutions of the serum of cows treated for months with intravenous and subcutaneous injections of living cultures and cultures heated at 62°C. The mutant was readily clumped in this serum in high dilutions. The agglutination of the (a) or original strain differed from that of the mutant or (b) strain in that a coherent mucoid disc formed in the bottom of the agglutination tube up to 1/10 dilution. In higher dilutions no clumping of any kind occurred. The serum from the cows treated with the original or parent strain protected guinea pigs receiving a surely fatal intraperitoneal dose of both original and mutant strains in doses of 0.005 to 0.01 cc. The serum of untreated cows failed to protect in doses up to 2 cc. With exceptions given below the original (a) strain has maintained its characters in agar slants kept at about 40°F., provided drying out was avoided and the transfer made from the bottom of the tube where some condensation water is always kept. The mutant

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[1] Smith, T., and associates, *J. Exp. Med.*, 1927, xlvi, 123–166.

Lebard[2] and the following year by Pfaundler[3] and called by him the thread reaction. With this method the clumping of (a) or disc formation was observed at a somewhat earlier period of the immunization process than when the usual method was employed. Several points need consideration when this method is used. In the first place bacteria are exposed to the agglutinins during multiplication. In the second place, the few bacteria introduced at the start are exposed to the full strength of the agglutinin. This gradually diminishes as the bacteria multiply and a point is reached when the agglutinin is entirely used up and the bacteria continue multiplying without clumping.

It was thought that by incubating the bacteria in various dilutions of serum in bouillon in the hanging drop some differences between (a) and (b) in the mode of agglutination might be noted. Thus far this has not been realized. Both (a) and (b) in suitably concentrated immune serum-bouillon grow in the form of interlacing chains of bacilli, each colony forming a distinct circular lacework of chains. Only when the agglutinins are exhausted is this form concealed and modified by diffuse growth. It was thought that this method of allowing growth in serum-bouillon might be useful in the absorption of agglutinins and the following experiments were carried out.

Direct and Reciprocal Absorption of (a) and (b) Agglutinogen in (a) Cow Serum.—When a 1/10 dilution of serum in bouillon was inoculated with a loop of Strain (a) and (b) and incubated the following phenomena were observed after 24 hours.

Strain (a) in Immune Serum-Bouillon.—The fluid is nearly clear. In the bottom is a compact disc-like mass, not disrupted by shaking. The fluid is considerably clouded by the shaking.

Strain (a) in Normal Serum-Bouillon.—Heavy clouding without any coherent deposit.

Strain (b) in Immune Serum-Bouillon.—Growth only in bottom of tube. Fluid clear. When shaken, the fluid becomes heavily clouded with flakes. No disc-like coherent mass. The upturned growth soon subsides.

Strain (b) in Normal Serum-Bouillon.—After 24 hours general

[2] Ledoux-Lebard, *Ann. Inst. Pasteur,* 1897, xi, 909.
[3] Pfaundler, M., *Centr. Bakt., I. Abt.,* 1898, xxiii, 9, 71, 131.

clouding with a lumpy deposit, easily broken up into smaller lumps, and general cloudiness.

In the course of the following 3 or 4 days there was increasing cloudiness in the immune serum-bouillon tube of (a). The gelatinous disc did not disappear. In the (b) tube the fluid remained clear. All growth took place in the bottom. Evidently in (a) the agglutinins were quickly used up and multiplication went on as in ordinary bouillon. After a week or longer the amount of growth in all tubes was much the same. Although it was chiefly a bottom growth in the (b) tubes, shaking produced a heavy turbidity.

To determine the effects of growth on the fate of the agglutinins, cultures in serum-bouillon were passed through Berkefeld filters. About 0.1 per cent dextrose and 10 per cent distilled sterile water were added to the filtrate to make up for losses and to effect a reduction of the pH through the action of the culture on dextrose. Such filtered fluid was inoculated with both (a) and (b) strains with the following outcome in 24 hours.

1. Serum-bouillon a-a showed no coherent disc-like deposit. The fluid was uniformly clouded with slight, easily suspended deposit.

2. Serum-bouillon a-b had only a bottom growth. This was granular and lumpy when suspended after shaking.

3. Serum-bouillon b-a had a firm, disc-like deposit. Fluid still clear.

4. Serum-bouillon b-b had a granular deposit and clear supernatant fluid.

In 48 hours (3) had become clouded. In 6 days (1) had become heavily clouded. No coherent growth in bottom. (2) was finely clouded with some surface growth. Deposit consists of lumps up to $1\frac{1}{2}$ mm. in diameter. Heavily turbid after shaking. (3) had become moderately clouded with persistent disc. (4) liquid still clear. When shaken the fluid cloudy with clumps up to 3 mm. in diameter.

A second successive filtration with the same additions as after the first filtration was carried out, the fluid tubed in 5 cc. amounts and inoculated with (a) and (b) strains. In the b-a-b tubes a deposit appeared but the supernatant fluid remained clear. When the tubes were shaken the clouding was much less pronounced than in the original cultures. Clumping in groups of 10 to 20 rods was demonstrated with the microscope. The a-b-a tubes were uniformly clouded without deposit. A microscopic examination showed no clumping.

A third filtration was carried out and the filtrate inoculated with (b). The b-a-b-b tubes showed growth in the form of a deposit. The fluid remained clear as heretofore. Even after shaking these tubes were clear on the following day.

The successive cultures in filtrates of serum-bouillon brought out the following facts: The (a) agglutinin was removed by the (a) but not by the (b) type. The (b) agglutinin was present in such amounts that three successive growths of (a) or (b) failed to remove it entirely. The (a) agglutinin forms a very cohesive mass of bacteria which is not broken up by repeated and vigorous shaking. In a normal NaOH solution the mass tends to soften and disintegrate and nearly disappear. In 5 per cent acetic acid it shrinks into a tough leathery membrane of much smaller bulk. Tested with the usual agglutinin technique, the b-a-b filtrate produced only a slight clumping of (b) in a dilution of 1/2 which was equivalent to a 1/20 dilution of the serum itself, thus indicating that the (b) agglutinin had been nearly used up. As a further control a normal cow serum was treated in the manner described, *i.e.* diluted with bouillon 1:10 and inoculated with Types (a) and (b). The (a) fluid became heavily clouded with the usual deposit. When shaken the fluid became more heavily turbid, without any clumping. A disc was not formed. The (b) fluid was at first clear with a bottom growth. Later the fluid became clouded as if the agglutinin present had been used up.

The above cultures were passed through Berkefeld filters and after adding 0.1 per cent dextrose inoculated with (a) and (b). (a) became uniformly and heavily clouded. In (b) a heavy deposit of clumps formed and the clouded supernatant fluid contained both clumps and free forms. Even in normal cow serum-bouillon the (b) agglutinins survived one multiplication of the bacilli.

The Direct and Reciprocal Absorption of Protective Antibodies.—The cow sera A and B tested for agglutinins as described above were also used in the following studies to determine their protective value. Cow A treated with living cultures and Cow B treated with heated cultures of (a) furnished sera of nearly the same protective titer. B was slightly higher and was capable of keeping alive guinea pigs receiving the surely fatal dose (usually $1\frac{1}{3}$ to $1\frac{1}{2}$ the minimum fatal dose) in amounts of 0.005 cc. to 0.01 cc.

The following experiment was carried out twice with entirely concordant results. Only the second is given.

20 cc. tubes of bouillon containing 10 per cent by volume of the immune serum of B were prepared. These were inoculated with B. coli (a) and (b). For controls a tube not inoculated was carried along with them and in addition a strain of B. coli (1085a) with capsule but without any agglutinative relation to (a). After 50 hours incubation the four tubes were refrigerated and filtered next day through separate Berkefeld filters. The filtrates were tested on guinea pigs to determine protective capacity. Each filtrate was mixed with 24 hour bouillon cultures of B. coli (a) and injected into the peritoneal cavity of guinea pigs. In the tests the weights of the guinea pigs were maintained between 350 and 375 gm. The bouillon for culturing was the same throughout. The culture dose was about $1\frac{1}{4}$ to $1\frac{1}{2}$ times the surely fatal dose. The outcome of the test was as follows:

The control serum-bouillon (incubated and filtered with the rest) protected in 0.5 cc., not in 0.3 cc. and 0.4 cc. doses[4]

TABLE I.

The Effect of Multiplication of B. coli (a), (b) and x in 10 Per Cent Immune Serum-Bouillon (a).

Serum used	Per cent serum in bouillon	Growth in serum-bouillon	Minimum protecting dose of serum
			cc.
Cow B	(Original undiluted serum)		0.01
"	10	Control	0.05
"	"	B. coli (a)	>0.30
"	"	" (b)	0.05
"	"	" x	0.07

The (a) serum-bouillon filtrate did not protect in 1.5, 2 and 3 cc. doses.[4]

The (b) serum-bouillon filtrate protected in 0.5 and 0.6 but not in 0.4 cc. doses.[4]

The control (1085a) serum-bouillon filtrate protected in 0.7 but not in 0.3 and 0.6 cc. doses. In Table I the serum present in the serum-bouillon is given in the last column.

The (a) strain of B. coli removed the protective substance so that 3 cc. serum-bouillon failed to lengthen the life period of the guinea pigs. It thus contained less than 1/6 of the protective substance in the control serum-bouillon. The (b) mutant removed practically none, the other strain of B. coli a little. This latter strain was nearly as

[4] To determine the actual serum present divide by 10.

virulent as the (a) strain. It will be noticed that the 10 per cent serum-bouillon control lost more or less during incubation and subsequent filtration. The serum in it protected in 0.05 cc. doses whereas the original serum protected in 0.01 cc. doses or less.

The Content of a (b) Antiserum in Agglutinin and Protective Antibody. —The serum of Cow C (treated with heated (b) mutant) agglutinated the mutant completely up to 1/1,280. At 1/10,240 the microscope still showed about one-third of the rods in clumps. Culture (a) was

TABLE II.

Protective Action of Immune (Cow) Serum (b).

Guinea pig No.	Dose of B. coli	Dose of serum	Result
	cc.	cc.	
1 (control)	0.06 (a)	—	Dies in 6 hrs.
2	0.07 (a)	0.5	" " 9 "
3	0.07 (a)	1.0	" " 9 "
4	1.3 (b)	—	" " 6½ "
5	1.4 (b)	0.01	" " 16 "
6	1.4 (b)	0.02	Lives

TABLE III.

The Effect of Multiplication of B. coli (a) and (b) in 10 Per Cent Immune Serum-Bouillon.

Guinea pig No.	Dose of B. coli (b)	Dose of 10 per cent serum-bouillon	Result Dies in	Amount of serum (calculated)
	cc.	cc.	hrs.	cc.
1	1.3	—	9	—
2	1.4	2 (a)	24	0.2
3	1.4	2 (b)	23	0.2
4	1.5	—	14±	—
5	1.5	3 (a)	14±	0.3
6	1.5	3 (b)	14±	0.3

slightly acted on in low dilutions only. Thus in a 1/2 or a 1/4 dilution the microscope showed chains of 4 to 8 rods among free individuals. About one-fourth of all rods were in short chains. No macroscopic agglutination was recognizable however in these or in the higher dilutions.

The protective action of the serum of Cow C was tested against

both the original *B. coli* (a) and its mutant (b).   Omitting the numerous tests for determining the minimum fatal doses of both (a) and (b) and the neutralizing dose of serum we present the final tests in Table II.

It will be seen from Table II that the serum of Cow C treated with mutant (b) failed to protect guinea pigs towards (a) in a dose of 1 cc.[5] whereas 0.02 cc. protected against the surely fatal dose of (b).   As has been stated, the (a) serum of Cow B protected against the surely fatal dose of both (a) and (b) in the same small amount of 0.01 cc.

To determine the absorptive capacity of (a) and (b) respectively with reference to the (b) immune serum a 10 per cent serum-bouillon was inoculated with (a) and (b), incubated for 46 hours and filtered. The filtrates were tested for residual protective substances on guinea pigs.

It will be seen from Table III that enough antibody had been removed by both (a) and (b) strains so that 0.3 cc. serum did not even retard death.   Higher concentrations were not tried because of the bulk of the serum-bouillon filtrate to be injected.   Unfortunately a control serum-bouillon was not carried through, but judging from the result given in Table I we may allow the surely protective dose of the serum-bouillon itself to be 5 times 0.02 cc. or 0.1 cc. serum.

DISCUSSION.

The experiments described point to the existence of two agglutinins, one directed towards the capsule and visualized in the mucoid disc-like mass formed during growth in serum-bouillon, the other directed against the naked bacteria represented by mutant (b).   The (a) agglutinin is produced *in vivo* with difficulty, the (b) agglutinin readily and abundantly.   The (a) antigen produces both (a) and (b) agglutinins, the (b) antigen only (b) agglutinins.   The mutant (b) fails to remove (a) agglutinins while multiplying in (a) serum-bouillon whereas (a) does.   These actions are visualized in the agglutinin disc which is absent in culture filtrates of serum-bouillon following the growth of (a) but is still present after the growth of (b).

The protective antibodies, as tested on guinea pigs by mixing serum and living culture and injecting into the peritoneal cavity, follow closely the agglutinins.   The (a) serum protects against both (a) and

[5] Higher doses not tried.

(b) forms of the homologous *B. coli* strain, the (b) serum only against (b). Both (a) and (b) strains remove or bind about the same amount of protective antibody in the (b) serum of Cow C. The abundance of (b) agglutinins produced by (a) in Cows A and B is shown by the fact that three repeated crops of *B. coli* in serum-bouillon failed to exhaust the fluid of all (b) agglutinin. A similar abundance of (b) agglutinin was produced in Cow C treated only with mutant (b).

In the preparation of antisera both living and heated (62°C.) cultures of the (a) form were of equal value when injected into cows. The few animals used do not permit generalizations, but it appears that the heated cultures were less injurious to the animal and produced antibodies more promptly perhaps because of the much larger numbers of bacilli that could be injected. Attention is called to the fact that *B. coli* is able to multiply abundantly in the first and second serum-bouillon filtrates of its own growth, less so in the third. After each filtration about 0.1 per cent dextrose was added to lower the pH of the culture fluid through the formation of acid by *B. coli*.

The formation of a coherent viscid disc-like mass as a result of agglutination appears to be associated with capsulated bacteria.

Wadsworth and Kirkbride[6] in testing a pneumococcus serum of Type III observed a prompt agglutination in a 1/1 concentration. "Within 15 to 30 minutes . . . . a loose cap had formed similar to those often seen in tests with Type I and Type II." Clumping was seen no higher than in 1/10 dilutions. Coulter[7] in preparing immune rabbit sera against Friedländer strains observed instant coarse flocculation settling down to a compacted disc in bottom of tube in serum dilutions of 1/1 and 1/5. Small and Julianelle[8] observed the same phenomenon in the agglutination of various strains of *B. mucosus capsulatus*. Avery and Morgan[9] describe antipneumococcus sera when acting on the specific soluble substance of Type II as producing in low dilutions a compact disc-like precipitate, in higher dilutions only flocculation.

The difficulty of producing or failure to produce antibodies towards capsulated strains has been observed and commented on by many working with such forms for the past 30 years. It would seem that

[6] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1917, xxv, 629.

[7] Coulter, C. B., *J. Exp. Med.*, 1917, xxvi, 763.

[8] Small, J. C., and Julianelle, L. A., *J. Infect. Dis.*, 1923, xxxii, 456.

[9] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.

such antibodies can be produced by using large, resistant animals and continuing the treatment over long periods. It may seem advisable to compare the horse and the cow with respect to this problem.

It has been noted in a previous paper[10] that the immune serum prepared with the *B. coli* strain (a) failed to protect guinea pigs against certain bovine types of *B. coli* and did protect against others but in doses about 50 times larger than that required against the homologous strain. Recently a highly viscid strain of *B. coli* was isolated from the spleen of a presumably normal guinea pig. The strain failed to ferment saccharose, being in this respect like (a). The viscidity was extreme. Cobweb-like threads several feet long were brought out of the culture tube with the loop and had to be burned off. Early mutation did not take place on agar plates. The intraperitoneal minimum fatal dose of a 24 hour bouillon culture was 0.2 cc. 0.25 cc. culture plus 0.5 cc. serum of Cow B (heated (a) culture treatment) protected. In serum dilutions of 1/2 to 1/20 of Cow B the bacilli were clumped into a floating, gelatinous mass. A certain limited relationship between the capsular material of this form and that of the bovine form is thus demonstrated.

The value of relationships based solely on serologic determinations is somewhat impugned by the above results. If (a) and (b) had been isolated independently, (b) would have been regarded as different from (a) since (b) serum fails to agglutinate (a), to absorb (a) agglutinins and to protect animals against (a). On the other hand (a) serum protects against (b) and agglutinates (b) in high dilutions. Such serologic relationships of bacteria morphologically and culturally alike but obtained from different sources may be regarded provisionally as indicating mutation or degradation of one type or strain (mutant) from the other (original). The taxonomic value of agglutinins obviously must depend on what is agglutinated. The capsular material, being probably developed by parasitism, is the last character to be acquired and most easily lost, as is evidenced by the rapid mutation on agar plates, during which process the capsule disappears. Since the fermentation reactions of (a) and (b) are not changed, it seems reasonable to regard them as of more fundamental value in

[10] Smith, T., *J. Exp. Med.*, 1927, xlvi, 141.

this group in classification than what is denominated the type-specific substance, which seems to be related to or identical with the capsular substance.

It has been stated in a previous paper[11] that immune cow serum does not exercise any appreciable influence on the toxic effect of culture filtrates of (a) and (b) when mixed with them and injected into the jugular vein of calves. It would have been of interest to determine quantitatively if any toxin-neutralizing action could be attributed to the sera of highly immunized cows. Owing to the difficulty of obtaining calves in sufficient numbers and of the same breed at any one time, no accurate comparative quantitative toxin-neutralizing experiments have been made.

CONCLUSION.

The relation between a strain of *B. coli* and its mutant with reference to the production of agglutinins and protective antibodies may be expressed by the statement that the original strain when injected into cows develops antibodies both towards itself and the mutant whereas the mutant produces them only towards itself. The results point to the capsular substance as the material carrying virulence or, expressed somewhat differently, the factor which protects the micro-organism in the host.

[11] Smith, T., and Little R. B., *J. Exp. Med.*, 1927, xlvi, 123.

RECIPROCAL EFFECTS OF CONCOMITANT INFECTIONS.

III. THE INFLUENCE OF VACCINIA AND OF VACCINAL IMMUNITY ON THE REACTION TO INFECTION WITH EXPERIMENTAL SYPHILIS (INTRACUTANEOUS INOCULATION).

BY LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a previous paper of this series (1), the results of experiments were reported in which it was shown that in rabbits the efficiency of the reaction to infection with syphilis was markedly decreased by the intracutaneous inoculation of vaccine virus at the time of the intratesticular inoculation of *Treponema pallidum*, the ensuing syphilis being much more severe than in control animals. In other experiments (2), a state of vaccinal immunity at the time of syphilitic inoculation was associated with an increased effectiveness of reaction, the disease being very mild. Results of a similar nature were obtained in which the intratesticular inocula consisted of a mixture of *Treponema pallidum* and vaccine virus (2).

In all the experiments already reported, the syphilitic inoculation was made in one testicle. Our experience, as well as that of others with experimental syphilis, has shown that the circumstances under which the syphilitic infection is initiated are essential factors in determining the general character of the reaction and that, among these factors, the site and mode of inoculation are of particular importance. As an extension of the present study of concomitant infections, experiments were carried out in which another route of syphilitic inoculation was employed, namely, the intracutaneous. In one group of rabbits, vaccine virus was injected intracutaneously at the time of the *pallidum* inoculation but at a different site, while in a second, the animals were immune to vaccine virus when inoculated with syphilis. The results of the experiment comprising the study of the reaction to syphilis induced by intracutaneous inoculation under these two conditions are

reported in detail in the present paper; a preliminary report has previously been published (3).

Materials and Method.

As in the other experiments dealing with the effects of a concomitant vaccinal infection upon the syphilitic reaction, the Nichols strain of *Treponema pallidum* and the Noguchi strain of vaccine virus were employed. The experiment was begun on Feb. 14, 1927, and the materials used for inoculation were the same as in the other groups of rabbits inoculated intratesticularly on this date (1, 2). The syphilitic emulsion prepared from an actively developing testicular lesion contained from 1 to 3 motile spirochetes per microscopic field. The vaccinal material was obtained from an early vaccinal orchitis, and its activity was controlled by the intracutaneous and the intratesticular inoculation of 4 normal rabbits.

The experiment comprised 3 groups of 10 rabbits each. The first group was inoculated with 0.2 cc. of the syphilitic emulsion intracutaneously on the dorsal surface of the prepuce, and immediately thereafter, the vaccinal emulsion (0.2 cc.) was injected intracutaneously on the side of the body while a similar amount was applied to an adjacent scarified area. The skin areas had been shaved just before these procedures were carried out.

The second group of rabbits was immunized to vaccine virus 30 days before inoculation with syphilis. Each animal reacted in a typical manner to the intracutaneous vaccinal injection and a second injection, 10 days after the first, was followed by no visible reaction. The syphilitic inoculation of these animals consisted in the injection of 0.2 cc. of the *pallidum* emulsion intracutaneously on the dorsum of the prepuce, as in the first group of animals.

The third group of the experiment comprised the syphilitic controls, that is, normal rabbits inoculated in the same site and with a similar amount of the *pallidum* emulsion as in the first and second groups.

The rabbits employed were young adult male animals approximately 8 months of age, and were carefully matched as to breed and color. They were separately caged throughout the experiment and were fed the usual diet of hay, oats, and cabbage.

The period of observation was 4 months during which time each rabbit was examined at frequent intervals in order that the course of the disease might be carefully followed. The syphilitic reaction was studied upon the same basis as in the former experiments already referred to (1, 2). Among the significant phenomena of the infection especially considered were the following: the incubation period, the character and duration of the primary lesion, the incidence, time of appearance, number, character, and persistence of generalized lesions, and finally, the resolution and healing of lesions or the initiation of the period of latency.

Although lesions of the testicles, tunics, epididymis, and vas deferens are, strictly speaking, generalized manifestations under the conditions of intracutaneous inoculation, they are of a somewhat different order than lesions of the perios-

teum, bone, skin and mucous membranes, and the eyes, and consequently have been separately considered. As has been found with the intratesticular route of inoculation, the testicles offer extremely favorable conditions for the growth of spirochetes, and in certain rabbits inoculated intracutaneously an orchitis may be the only generalized manifestation detected. Furthermore, no distinction has been made in the present discussion between lesions of the testicles and those of the tunics, epididymis, and vas since the latter structures are frequently involved in an extension of the testicular process.

In analyzing the results, special attention has been paid to the time relations of the successive phenomena of reaction. The term "focal distribution" or "focal rate" as applied to generalized lesions, refers to the number of such lesions per rabbit as determined by clinical examination. The actual rate includes only those animals which developed generalized lesions, while the relative rate refers to all the animals of a group. In estimating the distribution of lesions for group comparisons, the relative rate gives a fairer impression of the extent of the lesions and furthermore, avoids the impression which would be obtained by the chance inclusion in any group of an individual animal with unusually severe syphilis.

RESULTS.

The results of this experiment are summarized in Tables I to III and in Text-figs. 1 and 2. The figures given in Tables I and II represent group values. The incidence of the various phenomena of the syphilitic infection chosen for comparison are given in Table I, together with the actual and relative focal distribution rates of generalized lesions. Table II contains the mean time of occurrence of these phenomena, while Table III lists the time of appearance of all generalized lesions in 2 day intervals as estimated from the date of inoculation. The course of the infection in the 3 groups of rabbits, that is, the animals inoculated with *Treponema pallidum* and vaccine virus, the vaccine-immunes, and the controls is illustrated graphically by the curves of Text-fig. 1 in which the abscissæ represent the animal incidence of the syphilitic phenomena and the ordinates the time in days from the date of inoculation. These curves bring out a characteristic feature of experimental syphilis, namely, that while the events of the infection are successive, they also to some extent overlap each other. The curves of Text-fig. 2 which represent the appearance of generalized lesions in 6 day intervals are compiled from the figures in Table III; an interval of this length was chosen in order to avoid the irregularities of very short periods such as are given in the table.

TABLE I.

Incidence of Various Phenomena of the Syphilitic Infection and the Focal Distribution of Generalized Lesions.

Animal group	Number of rabbits	Chancre	Generalized lesions		
			Incidence	Focal distribution	Focal distribution
		per cent	*per cent*	*actual*	*relative*
Control....................................	10	100.0	50.0	4.4	2.2
V. V..	10*	100.0	88.8	6.0	5.3
V. V. Im..............	10	100.0	30.0	1.0	0.3

In this and other tables:

C = syphilitic controls.

V. V. = rabbits inoculated with vaccine virus.

V. V. Im. = rabbits immune to vaccine virus.

* One animal in this group which died of an intercurrent infection 98 days after inoculation has been included in the analysis of the primary but not of the generalized lesions.

TABLE II.

Mean Time of Occurrence of the Various Phenomena of the Syphilitic Infection as Estimated in Days from the Date of Inoculation.

Animal group	Chancre	Generalized lesions					
		First All lesions	First Not testicular	First Testicular	Last	Mean of all	Duration Active period
	days	*days*	*days*	*days*	*days*	*days*	*days*
Control...................	27.1	75.2	78.0	94.0	101.2	88.4	26.0
V. V......................	17.4*	68.1	67.1	72.5	92.6	75.3	24.5
V. V. Im.................	37.8	85.3†	75.0	106.0	85.3†	85.3†	0

* The omission from this calculation of the animal which died 98 days after inoculation would diminish the mean value for this group to 16.0 days.

† Since only 1 lesion developed in each of 3 vaccine-immune rabbits, the mean values for the time of appearance of the first and last lesions as well as the mean time of all lesions are identical.

TABLE III.

Time of Appearance of the Generalized Lesions as Estimated from the Date of Inoculation.

Time interval	Number of lesions			Time interval (cont.)	Number of lesions (cont.)		
	Controls	Vaccinated	Vaccine-immunes		Controls	Vaccinated	Vaccine-immunes
days				*days*			
40	0	1	0	82	0	0	0
42	0	0	0	84	3	4	1
44	0	0	0	86	0	1	0
46	0	0	0	88	0	1	0
48	0	0	0	90	3	1	0
50	0	2	0	92	0	2	0
52	0	2	0	94	4	2	0
54	0	0	0	96	0	0	0
56	0	1	0	98	4	0	0
58	0	0	0	100	0	0	0
60	0	1	0	102	1	0	0
62	0	7	0	104	0	0	0
64	0	0	0	106	1	1	1
66	1	3	1	108	0	0	0
68	0	0	0	110	0	0	0
70	1	4	0	112	0	0	0
72	2	2	0	114	0	0	0
74	0	1	0	116	1	1	0
76	0	4	0	118	0	0	0
78	0	2	0	120	1	1	0
80	0	4	0	Total....	22	48	3

DISCUSSION.

In the analysis and discussion of the results of this experiment, the various phenomena of the syphilitic infection have been taken up in the order of their occurrence. The disease picture is thereby simplified and variations in the behavior of the vaccinated and vaccine-immune animals as compared with the controls are the more readily appreciated.

There are certain features of the disease induced by intracutaneous inoculation that should be kept in mind not only in analyzing the present results, but in comparing them with those obtained in other experiments in which the intratesticular route of inoculation was

employed (1, 2). In general, it may be said that the disease as a whole is less certain, and one must take into account the fact that the manifestations of the infection as regards incidence, number, and time relationships are more variable than is the case with intratesticular inoculations. The questions of comparative amounts of inocula, the inherent differences of the inoculated tissues as affecting the growth, multiplication, and dissemination of spirochetes, as well as the more obscure differences of immune reactions that may be presumed to exist in such organs as the skin and the testicle are some of the factors to be considered in a comprehensive comparison of the reaction to infection induced by these two routes of inoculation. It will suffice

TEXT-FIG. 1.
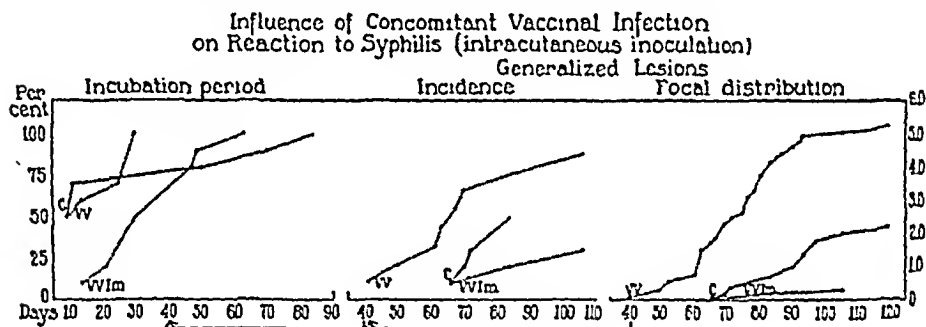
for the purpose of the present discussion, however, to indicate briefly some of the more important differences in the disease under these conditions.

With an infection of average severity induced by sheath inoculation, the incubation period of the chancre in the majority of a group of 5 or 10 animals is about the same as that of an orchitis, or it may be slightly shorter, owing possibly to the fact that the initiation of a cutaneous reaction is more easily recognized. But there are usually certain animals in which the development of the chancre is considerably delayed and this irregularity is more pronounced both as regards incidence and time than is the case with the testicular primary reaction in a group of rabbits of similar size. Furthermore, the chancre is prone to relapse, and while this tendency also occurs with testicular lesions, it is more frequent and usually more marked. The incidence of generalized lesions is generally lower with the intracutaneous than

with the intratesticular route of inoculation, there are fewer lesions, and the time of their appearance is delayed. It should also be noted that the tendency toward the persistency of lesions noted in connection with the chancre may extend to the secondary manifestations as well.

Chancre.—All animals in each group developed a primary lesion, as shown in Table I. As far as the majority of animals were concerned, there was no difference in the incubation periods of the vaccinated and control rabbits (Text-fig. 1). In 70.0 per cent of the controls, the
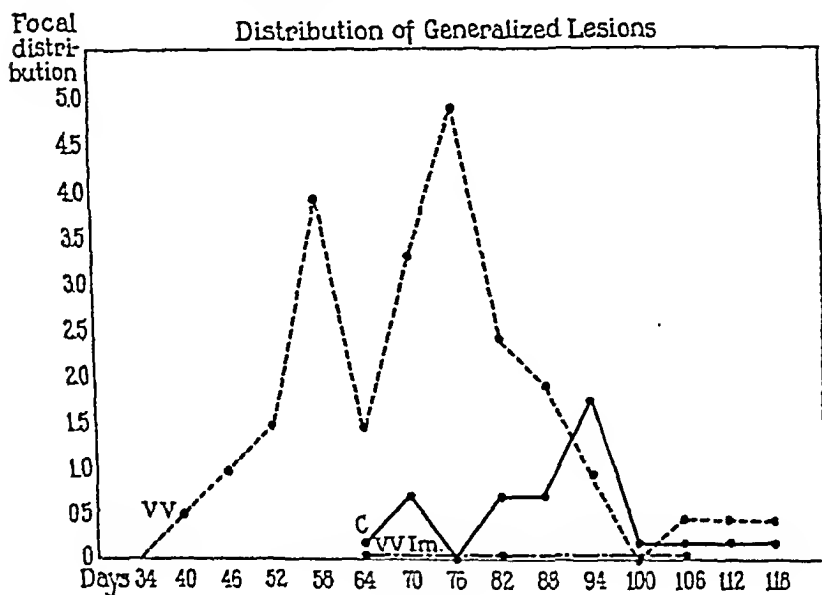
TEXT-FIG. 2.

chancres developed in the mean time of 9.6 days while in 60.0 per cent of the vaccinated animals, the mean time was 9.8 days, or 12.0 days in the case of 70.0 per cent of the vaccinated rabbits. But with the remaining animals in each group the mean incubation time was 68.0 days for the 3 controls as contrasted with 28.8 days for the 4 vaccinated animals or 30.0 days if 3 vaccinated animals are considered. If the comparison of incubation time is made from the group standpoint, therefore, the development of primary lesions was more uniform in the vaccinated animals than in the controls. This significant fact is

illustrated graphically by the curves in Text-fig. 1. The mean incubation values reflect the time differences in chancre development of the minority of the animals in each group, the figures being 27.1 days for the controls and 17.4 days for the vaccinated group, a difference of 35.8 per cent.

On the other hand, the primary incubation period was prolonged to 37.8 days in the case of the vaccine-immune animals, or 10.7 days longer than with the controls, a difference amounting to 39.5 per cent. The curve for the vaccine-immune group in Text-fig. 1 representing the initial stage of the disease, however, is again more regular than the curve for the controls, and the position of its last portion in advance of the curve for the controls suggests that in some instances the initial reactivity or sensitivity of rabbits to the intracutaneous inoculation of *Treponema pallidum* may be increased by an immunity to vaccine virus as was also suggested by the results obtained with the intratesticular route of inoculation (2).

Generalized Lesions.—The incidence of lesions which developed at sites other than that of the inoculation area is shown in Table I. Among the controls, generalized lesions were detected in 50.0 per cent of the animals, but the incidence in the vaccinated group was much higher, that is, 88.8 per cent, while it was only 30.0 per cent among the vaccine-immunes.

The distribution of generalized lesions in the three groups was in accord with their incidence as shown by both the actual and relative focal rates (Table II). As compared with the controls, the rates for the vaccinated groups were higher and for the vaccine-immunes much lower. The percentage values of the actual and relative rates of the experimental groups in terms of the control figures bring out clearly the extent of these differences.

Distribution Rates of Generalized Lesions.

Group	Actual	Per cent of control value	Relative	Per cent of control value
Controls	4.4		2.2	
V. V.	6.0	+36.4	5.3	+140.9
V. V. Im.	1.0	−77.3	0.3	−86.4

The generalized manifestations may further be compared upon a time basis, the values for which are given in Table II. As far as the mean time of appearance of the first secondary lesions is concerned, the vaccinated groups antedated the controls by 7.1 days while there was a delay of 10.1 days beyond the control value in the case of the vaccine-immune animals. If the comparison be made with the values of all generalized lesions except those of the testicle, the result is practically the same but the differences are somewhat greater in the case of the testicular lesions alone (Table II), that is, 21.5 days earlier than the controls for the vaccinated group and 12.0 days later than the controls for the vaccine-immune animals. The most interesting point in regard to the first testicular lesions, however, is that they occurred at about the same mean time as the other initial secondary manifestations in the vaccinated group, while they developed much later in the controls and the vaccine-immune animals. Thus, differences in the time of appearance of the first testicular lesions and of other generalized lesions as compiled from the values given in Table II are: vaccinated group, 5.4 days; controls, 16.0 days; vaccine-immune group, 31.0 days.

In regard to the mean time of appearance of the last generalized lesions, the largest value occurred with the control group, that is, 101.2 days as compared with 92.6 and 85.3 days for the vaccinated and vaccine-immune groups respectively. But it must be remembered that these figures are mean values, and reference to Text-fig. 1 shows that generalized lesions continued to appear in the vaccinated group toward the end of the observation period as they did in the controls. In the case of the immune rabbits, however, only 3 generalized manifestations were detected, 1 each in 3 animals, so that the mean values for the appearance of the first and last lesions are identical. It should be noted, however, that the last of these lesions to develop appeared 14 days before the last lesions observed in the other 2 groups as shown by the curves in Text-fig. 1.

The duration of the active period of generalized lesions as calculated from the mean values of the time of appearance of the first and last lesions was practically the same for the controls and the vaccinated groups, 26.0 and 24.5 days respectively, but with the vaccine-immunes,

the period of eruptive activity cannot be calculated on this basis, for as has just been mentioned, the 3 lesions in this group developed in 3 different animals (Table II, Text-fig. 1).

As far as the mean time of appearance of *all* generalized lesions is concerned, the value for the vaccinated rabbits is smaller than for the controls, that is, 75.3 and 88.4 days respectively (Table II), which suggests that a large number of lesions in the former group developed at an earlier period. That this was actually the case is shown by the distribution curves of Text-fig. 2 in which the appearance of lesions is plotted upon a time basis. In drawing these curves, the relative focal rates of generalized lesions have been used, so that one also obtains an idea of the extent of the eruptive period in the 3 groups of animals. It is at once evident by comparing the shape and height of the curves for the vaccinated and control groups that a large number of secondary manifestations had developed in the vaccinated animals before any were detected among the controls, indicating a more prompt and vigorous reaction on the part of these rabbits as, it will be remembered, was also the case with the primary lesion. Furthermore, the height of eruptive activity in the vaccinated group occurred from 70 to 82 days after inoculation, while with the controls it was from 82 to 94 days. These differences account for the magnitude of the values for the mean time of appearance of all generalized lesions.

An interesting feature of the general character of syphilitic infections is illustrated by these curves. The development of many generalized lesions is frequently followed by a more or less prompt cessation of such activity, while if fewer lesions are distributed over a similar or somewhat longer period, the phase of eruptive activity is apt to continue for some time. Among the control animals of this experiment, for instance, secondary lesions first appeared 64 days after inoculation, and they continued to develop at approximately the same rate until the 100th day and from then on at a much lower rate to the 120th day when the observations were discontinued.

With the vaccinated group, on the other hand, the first generalized lesions appeared on the 40th day after inoculation. During the next 18 days a large number of lesions developed, and although this phase was followed by a brief fall in eruptive activity, it was succeeded by a period of 30 days during which a great many lesions developed. In

the last fortnight of the experiment, a few lesions continued to appear, and although the focal rate was much lower than it had previously been, it was definitely higher than that of the control group at this time. There are 3 points in particular in which this course of infection was unusual. In the first place, the initial phase of pronounced eruptive activity commenced unusually early and it continued for a considerable time at a very high rate and thirdly, there was a continuance of activity late in the course of the disease when one might expect a cessation of generalized lesions in view of the preceding course of events. It would appear, therefore, that the reaction of the vaccinated animals to the syphilitic infection was not only affected at the initiation of the phase of secondary manifestations, but for a considerable time thereafter and in an unusual manner.

As far as the vaccine-immune group is concerned, the 3 points indicated in Text-fig. 2 show in contrast to the curves representing the vaccinated and control groups, the very slight extent and degree of the generalized phase of the infection. This feature is also illustrated in Text-fig. 1 by the low height of the curve representing the focal distribution of secondary lesions among the vaccine-immune animals.

In discussing the generalized phase of the infection, the question of the particular site or tissue involved has been mentioned only in connection with the time of appearance of the first generalized lesion, that is, as to whether the site of the first lesion was or was not testicular. The relative incidence of testicular involvement as compared with other secondary lesions is shown in the following table:

Incidence of Generalized Lesions in Different Sites.

Group	Testicle	Bone and periosteum	Skin and mucous membranes	Eyes
	per cent	*per cent*	*per cent*	*per cent*
Control	50 0	40 0	10.0	0
V. V.	55 5	77 7	22 2	0
V. V. Im.	10 0	20 0	0	0

All control rabbits which developed generalized manifestations developed testicular lesions as well, but there were 3 vaccinated and 2 vaccine-immune rabbits which did not. On the other hand, there

were 1 vaccinated and 1 vaccine-immune animal, as there was 1 control, in which a testicular lesion was the only generalized manifestation. These results should be considered in connection with the extent of involvement of the above sites.

Number of Lesions in Different Sites.

Group	Testicle		Bones and periosteum		Skin and mucous membranes	
	No.	Per cent of all lesions	No.	Per cent of all lesions	No.	Per cent of all lesions
Control........................	6	27.3	15	68.2	1	4.5
V. V............................	6	12.5	39	81.2	3	6.3
V. V. Im......................	1	33.3	2	66.7		

From these two analyses, it is evident that while the incidence of testicular involvement was the same in the control and vaccinated groups, the latter group showed a far greater number of bone and periosteal lesions. In addition, cutaneous lesions developed in 2 vaccinated as compared with 1 control animal. The relative proportion of testicular and bone lesions in the 2 groups as shown by the percentage values above is a further indication of the disturbed reaction of the vaccinated animals, a disturbance which is both quantitative and qualitative. In the vaccine-immune group, on the other hand, the general type of the reaction as judged by the percentage values of the various kinds of generalized lesions was similar to that of the controls, but the great difference in the actual numbers of lesions shows at once a quantitative difference in the reaction of the vaccine-immune animals.

Recovery.—The last point to be discussed is the condition of the lesions 4 months after inoculation. As with the intratesticular route of inoculation, there is a considerable variation at this time in a group of 5 or 10 rabbits with respect to the proportion of animals in which all lesions have healed, in which case the infection is said to be latent, and the number of animals in which lesions are still present. In this connection, it should be recalled that chancres are especially prone to relapse, and that with the intracutaneous route of inoculation, generalized lesions usually develop later than with an intratesticular inocula-

tion. The final observations in this experiment are summarized in the following tables in which the lesions are classified as healed, not healed, or active.

State of Primary Lesions 4 Months after Inoculation.

Group	Healed	Not healed	Active
	per cent	*per cent*	*per cent*
Control....................................	60.0	30.0	10.0
V. V......................................	33.3	22.2	44.4
V. V. Im..................................	60.0	40.0	

State of Generalized Lesions 4 Months after Inoculation.

Group	Healed	Not healed	Active
	per cent	*per cent*	*per cent*
Control....................................	50.0		50.0
V. V......................................	22.2		77.7
V. V. Im..................................	90.0		10.0

It is shown by these analyses that active primary and generalized lesions were present 4 months after inoculation in a greater proportion of vaccinated than control animals, while the reverse obtained with the vaccine-immunes. The results are of importance in that they are of the same general order as those already discussed in connection with the earlier phenomena of the syphilitic reaction, but their special significance lies in the fact that the effect of a concomitant vaccination or of a vaccinal immunity was sustained for so long a period.

From what has been said regarding the results of the experiments here reported, it is evident that intracutaneous inoculation with vaccine virus at the time of intracutaneous inoculation with *Treponema pallidum* was associated with a well marked disturbance in the syphilitic reaction, particularly as regards the incidence and time relationships of reactive phenomena, the relative frequency of occurrence of various manifestations, and the relative failure on the part of an early and pronounced generalized phase to reduce or prevent the continuance of eruptive activity. Judging from the comparisons made of the various criteria selected for analysis, it is clear that the efficiency of reaction in these animals was definitely lowered with the result that the infection was severe and prolonged.

With respect to the vaccine-immune rabbits inoculated intracutaneously with *Treponema pallidum*, on the other hand, the incidence and time relationships of reactive phenomena as well as the relative frequency of occurrence of various manifestations demonstrate very definitely that the syphilitic reaction was also affected, but in the direction of increased efficiency with the result that the infection was mild and comparatively short in duration.

Bearing in mind the fact that the syphilitic infection induced by an intracutaneous inoculation differs in several important respects from that resulting from the intratesticular route, the present results are in substantial accord with those previously reported (1, 2). In both instances, vaccine virus injected intracutaneously at the time of syphilitic inoculation was associated with a severe disease and likewise in both instances, a state of vaccinal immunity at the time of syphilitic inoculation was associated with a mild disease. Whatever may prove to be the mechanism by which these effects are brought about, the influence of the two conditions under discussion, that is to say, a concomitant vaccinal infection and a concomitant vaccinal immunity, was sufficiently pronounced to be perceptible in well defined modifications of the syphilis induced by two quite different routes of inoculation.

It is well recognized that the syphilitic reaction may be variously modified by a number of procedures, as for instance by different forms of treatment, surgical measures, and the method and route of inoculation. The results of these and previous experiments already referred to demonstrate that the reaction may also be affected by the presence of another infection as well as by the presence of an immunity to this second infection. It will be recalled, however, that in the case of vaccinia as compared with syphilis, the infection is of short duration and that immunity develops rapidly. It would appear, therefore, that the conditions present at the time of syphilitic inoculation constitute a factor of major importance in determining the course of infection.

Although little is known regarding the nature of the syphilitic reaction, there can be no doubt but that it involves many factors that are essentially expressions of functional activity, and from this standpoint it appears that the influence of a concomitant vaccinal infection or of a preexisting vaccinal immunity is mainly concerned with the

properties of host reaction and resistance. But the information regarding the question of concomitant diseases is meager, and the possibility that conditions affect the spirochetes, either directly or indirectly, must not be forgotten.

SUMMARY.

An experiment is reported in which was studied the effects of a concomitant vaccinal infection and of vaccinal immunity upon the reaction to syphilis in rabbits induced by intracutaneous inoculation.

The results obtained showed that the reaction was modified by both conditions. A vaccinal infection initiated at the time of syphilitic inoculation was associated with a defensive reaction of lessened efficiency, the ensuing syphilis being more severe than in control animals. A state of vaccinal immunity present at the time of syphilitic inoculation was associated with a reaction of heightened efficiency, the ensuing syphilis being very mild.

These results are in harmony with those obtained in other experiments in which the intratesticular route of syphilitic inoculation was employed.

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THE ACTION OF THE LEVADITI STRAIN OF HERPES VIRUS, AND OF VACCINE VIRUS IN THE GUINEA PIG.

SINGLE AND COMBINED EFFECTS.

By PETER K. OLITSKY, M.D., AND PERRIN H. LONG, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The guinea pig is susceptible to most strains of the virus of herpes, but there are certain ones, such as the Levaditi *souche* C, which, on intracerebral inoculation in this animal,[1] are inactive. Flexner[2] has shown that such differences are explainable on the basis of variations in virulence, weak strains being implanted on the cerebrum with difficulty and strong strains with ease. An interesting analogue to this condition may be found in such a widely different infectious agent as the filtrable virus of foot-and-mouth disease. Among types of this virus some may be found which are easily transferred to guinea pigs (Type A, for example),[3] while others are not.

The resistance of the guinea pig to some strains of herpes virus has been recently emphasized by Dmitrieff[4] and Rose and Walthard.[5] They consider that the behavior of the animal in this regard offers a possible solution to the problem of the etiology of epidemic encephalitis in man. Their idea is that the inability to transfer this disease from man to rabbits may be explained by a natural resistance such as that manifested by guinea pigs to herpes virus. Flexner,[2] on the other hand, has brought out the point that the guinea pig merely acts to discriminate strong from weak strains of herpes virus. Consequently the recognition of this factor does not aid in the solution of the etiology of epidemic encephalitis.

[1] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 233.

[2] Flexner, S., *J. Exp. Med.*, 1928, xlvii, 23.

[3] Olitsky, P. K., Traum, J., and Schoening, H. W., Report of the Foot-and-Mouth Disease Commission of the United States Department of Agriculture, 1928, *Tech. Bull. 76*.

[4] Dmitrieff, S., *Z. Hyg. u. Infektionskrankh.*, 1926, cvi, 547.

[5] Rose, G., and Walthard, B., *Z. Hyg. u. Infektionskrankh.*, 1925–26, cv, 645.

In this paper we intend to present the results of attempts to implant a weak strain of herpes virus on the cerebrum of the guinea pig by the use of special methods.   In some of the tests dermo- and neurovaccine viruses were used.   The experiments included a study of the nature of the barrier to implantation of weak strains, as well as one of immunity reactions to both weak and strong strains.

In carrying out the following tests, we employed as a sample of a weak strain of herpes virus the Levaditi C strain which, in agreement with previous findings,[2] has failed to infect a large number of guinea pigs after ordinary intracerebral inoculation.   In some tests this virus was used by itself, and in others in combination with the viruses of vesicular stomatitis of horses,[3] or of vaccinia, both dermovaccine and neurovaccine.[6]   Experiments of the latter sorts involved necessarily a study of the individual effects of the vesicular stomatitis and vaccine viruses in the guinea pig, so that the action of these when combined with the Levaditi strain could be properly understood.   For certain purposes, a strong strain of herpes virus was needed.   We then used the H.F.[1] and the J.B.[7] strains.

The methods employed in preparing suspensions of brain for inoculation, the manner of injection, and the observations of treated animals followed the procedures of Flexner and Amoss,[7] unless otherwise stated.

Survival of Herpes Virus (Levaditi Strain) in the Brain of the Guinea Pig.

The first series of experiments[8] related to a study of the period of survival of the Levaditi strain of herpes virus in the brain of the guinea pig.

For this purpose, a number of guinea pigs, as shown in Table I, were injected intracerebrally in one hemisphere, with controlled, active Levaditi virus, as contained in the cerebral tissue of rabbits dying from virus encephalitis.   The amount of active rabbit brain used for inoculation of the guinea pigs was, as a rule, 0.2 cc. of a 10 per cent saline suspension.   After various periods of time, the animals were killed and a fragment of forebrain, usually about 0.5 gm. in weight, was removed in a sterile manner from a region showing the track of the needle. A 10 per cent saline suspension of this fragment was made and 0.4 cc. inoculated intracerebrally into each of two rabbits.   The animals were then closely studied for any indication of virus encephalitis.   The brains of the rabbits which suc-

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[6] For two samples of neurovaccine placed at our disposal, we are indebted for one to Dr. Rivers, of The Rockefeller Institute, who had originally received the strain from Professor Levaditi, and for the other to Professor Levaditi himself.

[7] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

[8] Ether anesthesia was employed on animals in all experiments.

cumbed, or were etherized when moribund, were sectioned and stained, and examined for the characteristic lesions of infiltrative meningoencephalitis, and for

TABLE I.

Period of Survival of Levaditi Virus in Guinea Pigs' Brains; as Demonstrated by Inoculations of the Brain Tissue into Rabbits.

Series	Days after intracerebral injection of the guinea pigs	Effects on the rabbits
A	2	No reaction
	3	" "
	4	1 of 2 rabbits showed characteristic virus encephalitis; death on 4th day
	5	1 of 2 rabbits showed characteristic virus encephalitis; death on 5th day
	7	No reaction
	9	" "
B	1	2 rabbits showed characteristic virus encephalitis; death on 7th and 9th days respectively
	2	No reaction
	3	" "
	4	" "
	5	2 rabbits showed characteristic virus encephalitis; death on 9th and 11th days respectively
	6	No reaction
C	1	2 rabbits showed characteristic virus encephalitis; death on 5th day in one instance and moribund on 5th day in the other
	2	No reaction
	3	" "
	4	" "
	5	" "
	6*	1 of 2 rabbits showed characteristic virus encephalitis; death on 9th day

* In an additional experiment two guinea pigs from which the cerebral fragments were removed 9 days after inoculation of virus failed to reveal the presence of virus by the rabbit test.

the typical intranuclear inclusion bodies. Cultures of the brains in chopped meat medium and in dextrose broth were also made to rule out the action of bacteria. The results are given in Table I.

It will be noted in the experiments of Series A that the brains of guinea pigs inoculated with the Levaditi virus were not active on the 2nd and 3rd days after cerebral injection, but were active on the 4th and 5th days. Thereafter, to the 9th day after injection, the virus could not be detected by means of the rabbit test. Also in Series B, virus was obtained from the guinea pigs' brains on the 1st and 5th days after injection, but not on the 2nd, 3rd, 4th, and 6th days. In Series C, virus could be demonstrated by the rabbit test on the 1st and 6th days after inoculation, but not on the 2nd to 5th days, and in an additional experiment, not on the 9th day.

One may suppose that the Levaditi strain of herpes virus, after its introduction into the brain of the guinea pig, remained there for 24 hours after inoculation in a concentration sufficient to show activity when the inoculated area was transferred to the cerebrum of the susceptible rabbit. In other words, a passive transfer of the virus might have occurred, explaining the positive results at this time. Thereafter, up to about the 4th day the virus was not detected. During this period, we believe the virus to have scattered throughout the cerebrum, and since it again became demonstrable by the rabbit test on the 4th to 6th day after injection, it probably increased in amount during this time. Thereafter it was not again demonstrable, a fact which would imply its final neutralization or elimination. It is important to note that during all this time the guinea pigs appeared healthy, and those brains removed for injection into rabbits were free from distinctive histopathological changes.

The brain of a guinea pig which had been found to contain virus failed to produce virus encephalitis after 53 days glycerolation, unlike glycerolated rabbit brains in which the virus survives for an indefinite period. From this experiment one might perhaps infer that in the guinea pig brain the concentration of virus was less than in the rabbit brain.

Neutralization Tests with Serum.

The following tests were made to determine whether any neutralizing factor for herpes virus exists in normal guinea pig serum.

Four experiments were planned, all with the same methods.

Preparation of Virus.—The source of the Levaditi herpes virus was fresh cerebral tissue of a rabbit dying from 3 to 7 days after intracranial inoculation of the virus. A 10 per cent saline suspension was made which was filtered without pressure through one layer of filter paper, for the purpose of removing the larger particles. In one test the H.F. strain of virus was similarly prepared and used as a control.

Preparation of Serum.—Two or three full grown guinea pigs were bled by cardiac puncture. The blood was pooled and kept at 37°C. for about an hour until the serum separated from the clot, when it was centrifugalized. In some tests, part of the serum was heated at 56°C. for ½ hour to destroy complement; the remainder was used in a fresh state.

Plan of Test.—As a rule, five tubes were prepared with 0.1 or 0.2 cc. of the paper-filtrate virus. To the first tube, 2 cc. of saline solution was added (control), to the second and third, 2 cc. and 1 cc. of inactivated serum were added, and to the fourth and fifth, 2 cc. and 1 cc. of fresh serum. The mixtures were repeatedly shaken and placed at 37°C. for 1 hour, and then overnight in the ice box at 5°C. Rabbits were then inoculated intracerebrally with 0.35 to 0.4 cc. of each of the mixtures to determine the survival of the virus.

In addition to these experiments, another one was made in which suspensions of normal guinea pig brain were added to the virus instead of the serum.

To summarize the results, suspensions of cerebral tissue or inactivated serum from normal guinea pigs failed to neutralize the virus. With fresh, normal guinea pig serum, however, complete neutralization was effected in one experiment, the incubation period was protracted to 11 and 13 days respectively (as compared with 6 and 7 in the controls) in two others, and no neutralization occurred in the last. In the case of the H. F. strain of herpes virus, to which the guinea pig, as a rule, responds with encephalitis, neither inactivated nor fresh serum neutralized the virus. It may be concluded that the results, although not entirely consistent, suggest that fresh, normal guinea pig serum has a neutralizing action on the Levaditi virus.

Inoculation of Levaditi Virus Alone.

The next series of experiments involved attempts to produce encephalitis in guinea pigs with the Levaditi virus, by various special means. To obtain active virus, we employed fresh, non-glycerolated brains removed from rabbits either in the terminal stages of Levaditi-virus encephalitis or shortly after death.

The first method involved the inoculation into the brain of a large amount of virus (0.05 to 0.1 cc. of a 10 per cent rabbit brain suspension) into three apparently healthy, 1 week old guinea pigs. Young animals were used to ascertain whether they might be less resistant.

The second attempt included the subdural injection of massive doses of the virus, that is, 0.2 cc. of a 10 per cent rabbit brain suspension into each side of the forebrain of normal 250 gm. guinea pigs. It was believed that this saturation of the brain to the limit imposed by the operative technique might yield different results from the usual injection of 0.15 cc. into one hemisphere.

In the third instance, medium-sized guinea pigs were inoculated intraperitoneally with massive doses of Levaditi virus (5 and 10 cc. of the usual fresh brain suspension) and at the same time intracerebrally with the ordinary amount —0.15 cc. In this series the attempt was made to saturate the body with virus and reduce the general resistance.

The fourth test consisted of the intracerebral passage of the virus at 5 day intervals through three successive series of guinea pigs. We aimed at the enhancement of the virulence of Levaditi virus by repeated transfer of cerebral tissue at a period corresponding to the maximum concentration of the virus, as shown in the first set of tests. ·

In a fifth experiment, we made use of the method of Teague and Goodpasture[9] who reported increase in virulence of herpes virus after its inoculation into the tarred skin of guinea pigs. Seven young, white-furred guinea pigs were subjected to treatment with Holland tar. Acting upon the finding of Teague and Goodpasture, that the sensory nerves and posterior root ganglia corresponding to the area of skin treated are affected, we applied the tar as close to the head as possible. The liquid was painted on the back of the neck and upper posterior chest four times at 3 to 4 day intervals. At the end of the period, the skin was hairless and hypertrophic. 10 per cent saline suspensions of fresh rabbit brain containing active Levaditi virus were then rubbed into the scarified areas, and injected intracutaneously into five of the pigs; the remaining two served as controls. The virus treatment was repeated after 48 hours and again after 5 days.

In the sixth test the Levaditi virus, as contained in rabbit brains, was inoculated into the scarified corneæ of guinea pigs. Suspensions of scrapings of the cornea of these animals were inoculated into normal guinea pigs, in this way propagating the virus through five successive corneal passages, in twenty animals.

None of these six methods served to establish specific encephalitis in guinea pigs. Neither were any local specific lesions manifest in any instance, except in the one in which the virus was inoculated into the cornea. In the series of corneal inoculations, all guinea pigs

[9] Teague, O., and Goodpasture, E. W., J. Med. Research, 1923, xliv, 185.

of the first to the fifth passages showed a definite but mild kerato-conjunctivitis, from which they recovered without showing cerebral symptoms.

In view of the finding, however, recorded in the first experiment, namely, that the Levaditi virus increases in quantity in the brains of guinea pigs, another mode of definite implantation of the virus was still searched for, and one was found.

The procedure consisted merely in a combination of corneal and subdural inoculation of the Levaditi virus into the same guinea pig at different times.

Two guinea pigs were injected intracerebrally with 0.2 cc. of a 20 per cent saline suspension of fresh rabbit brain containing Levaditi virus. At the same time, the scarified left cornea was inoculated with the same suspension of virus. The next day the animals revealed definite keratoconjunctivitis. On the 3rd day after the first treatment, the animals were inoculated in the scarified right cornea with the corneal scrapings derived from a guinea pig with keratoconjunctivitis resulting from a corneal virus inoculation. On the 4th day both eyes of each animal exhibited keratoconjunctivitis. On the 6th and 7th days respectively, both guinea pigs became hypersensitive and showed circling movements, tremors, involuntary muscular contractions, urine retention, gnashing, and marked salivation. When moribund, they were killed and the brains removed for culture, histopathology, glycerolation, and for further guinea pig passages. In gross the brains were quite normal, except for a slight injection; the other organs were unaffected. Cultures of cerebral tissues gave no growth. Microscopic examination of the brains disclosed the severe, infiltrative meningoencephalitis characteristic of herpes-virus encephalitis in rabbits and guinea pigs. There were also present large numbers of intranuclear inclusion bodies typical of herpetic infection.

This experiment was repeated three times with the same outcome in each.

Thus, finally, characteristic herpes-virus encephalitis was induced in the guinea pig with the Levaditi strain. Moreover, the effects were obtained through seven successive guinea pig passages:[10] the first to the third by the special combination of subdural and corneal inoculations; the remaining passages by intracerebral injections only. On transfer of the suspensions of cerebral tissue to rabbits' corneæ or brains, the latter developed typical herpes-virus effects followed by

[10] After four to five successive passages in the guinea pig, with subdural inoculation only, the experiments were discontinued.

death from encephalitis. Furthermore, after the virus acquired its encephalitogenic property, the usual dose employed with strong strains (the H.F., for example) sufficed to induce the characteristic encephalitis in guinea pigs. Moreover, the encephalitogenic Levaditi virus behaved like strong strains in guinea pigs in that the kerato-conjunctivitis tended often to complete recovery. As is also the case with strong strains injected intracerebrally in guinea pigs, recovery would occasionally occur in an animal of a series which showed the characteristic signs of encephalitis.

Having succeeded in adapting the Levaditi C virus to guinea pigs by the double method of inoculation described, the concomitant effects of the viruses of vesicular stomatitis and of vaccinia were next tested.

Concomitant Effects of Levaditi Virus and Other Viruses.

Recent reports have shown the growth and survival of vaccine virus in tumors of mice and rats (Levaditi and Nicolau[11]), of Virus III, and vaccine virus in a transplantable rabbit neoplasm (Rivers and Pearce[12]), and the enhancement of the effects of syphilis in rabbits with vaccinia (Pearce[13]). Levaditi and Nicolau[14] have reported also on the concomitant action of herpes and vaccine viruses in rabbits, through which vaccine virus was rendered more active and made to induce characteristic encephalitis.

For experimental purposes the virus of vesicular stomatitis of horses, already described[3, 15] and vaccine virus were used. The latter was generously supplied by the New York City Health Department Research Laboratories.

Vesicular Stomatitis Virus.—This material consisted of a fixed guinea pig passage virus, propagated through several hundred animals. It was capable of inducing characteristic vesicles in the pads and corneæ of the animals 24 to 48 hours after inoculation, but was free from neurotropic action.

Vaccine Virus.—The virus consisted of calf lymph that was active in the skin, testicle, and cornea of guinea pigs, producing typical vaccinal lesions within 2 to 4 days after inoculation. This sample of virus was not neurotropic, failing to induce encephalitis after intracerebral inoculation of guinea pigs.

[11] Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, xxxvii, 443.

[12] Rivers, T. M., and Pearce, L., *J. Exp. Med.*, 1925, xlii, 523.

[13] Pearce, L., *J. Exp. Med.*, 1928, xlvii, 611.

[14] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1925, xciii, 3.

[15] Olitsky, P. K., *J. Exp. Med.*, 1927, xlv, 969.

Tests.—Different combinations of viruses were made, as follows: Levaditi C virus was injected intracerebrally in guinea pigs of all the series. In two of the series, vesicular stomatitis and vaccine viruses were also inoculated intracerebrally at the same time. In a third series, vesicular stomatitis virus was injected in the pads at the time the Levaditi virus was introduced into the brain; and in a fourth, vaccine virus was injected intracutaneously. In each instance the viruses by themselves were found to be active; the Levaditi virus in control rabbits, and the vesicular stomatitis and vaccine viruses in control guinea pigs.

The simultaneous injection of Levaditi virus in the brain and vesicular stomatitis or dermatotropic vaccine virus also in the brain, or in the skin, failed, on the other hand, to produce any cerebral effect.

In the next experiments two specimens of neurovaccine were used in additional tests on the combined action of the viruses. The neurovaccine was much more active than the dermatotropic sample in the skin, cornea, and testes of guinea pigs.

Characteristics of Neurovaccine Virus.—The samples of neurovaccine virus were alike in their action in animals.

In rabbits injected intracerebrally, the virus acted in a manner similar to the herpes virus. The symptoms shown by the inoculated animals were identical, except that in the case of neurovaccine virus the experimental disease was more severe; the first signs were manifest within 48 hours after inoculation, and death ensued, as a rule, within 72 to 96 hours. The histopathology of the brains consisted of infiltrative meningoencephalitis, but the characteristic intranuclear inclusion bodies of herpes encephalitis were absent.

The neurovaccine virus differs from herpes virus in the failure to show cross-immunity reactions. Rabbits recovered from keratoconjunctivitis induced by the H.F. II strain of herpes virus[16] were not resistant to later intracerebral injections of neurovaccine virus. Furthermore, the neurovaccine virus produced characteristic vesicular keratitis in rabbits and Guarnieri bodies were found in the lesions. Finally, the virus was active in the skin and testes of rabbits, in which typical vaccinal eruption or the orchitis was produced.

The effects of neurovaccine in guinea pigs differ from those in rabbits to a noteworthy degree. After intratesticular injection an orchitis developed on the 2nd day which tended to heal rapidly. No cerebral involvement followed. After intradermic inoculation, characteristic vesicles appeared on the 2nd day with no encephalitic complication. Corneal injection was followed by a keratoconjunctivitis on the 2nd day without cerebral involvement. Finally, intracerebral inoculation of neurovaccine virus was without any specific action. Thus the neurovaccine produced milder lesions in the guinea pig than in the rabbit, and in the guinea pig it was inactive in the brain.

[17] Flexner, S., *J. Exp. Med.*, 1928, xlvii, 9.

It is evident that the neurovaccine virus, although distinct from herpes virus, acts in rabbits and guinea pigs in a manner comparable to the Levaditi C virus itself. For, while it produces an encephalitis in rabbits, it does not do so in guinea pigs. It does, however, induce local lesions in guinea pigs after intradermal, corneal, and testicular injection, which are manifest, as a rule, 2 days after inoculation.

In combining neurovaccine virus with the Levaditi C strain of herpes virus in the effort to evoke an encephalitogenic effect of the latter in guinea pigs, it was believed that success might follow the injection of the two viruses at different times, so that both would eventually operate at the same period with maximum intensity. We had already determined this period for neurovaccine virus, namely, 2 days. Earlier in this paper, we have shown that the time of maximum activity of the Levaditi strain of herpes virus in the brains of guinea pigs is from 4 to 6 days after its intracerebral injection.

Combined Effects of Levaditi Herpes Virus and Neurovaccine Virus.

The following tests were made in a study of the concomitant effects of the Levaditi virus and neurovaccine virus.

In carrying out the experiments, when virus was to be injected in the brain, the usual dose of 0.15 cc. of a 10 per cent saline suspension of brain containing active virus was used, unless otherwise stated. For corneal and skin injections, the methods of Flexner and Amoss[8] were employed. For intratesticular inoculation of neurovaccine virus, 0.5 cc. of a 10 per cent rabbit brain suspension, containing active virus, was introduced into each of the testes after injury of these organs by repeated needle puncture. In each instance the specific activity of the viruses employed was determined by inoculation into corresponding tissues of susceptible control animals. The period of observation lasted for from 15 days to at least 1 month.

The combinations of viruses injected into guinea pigs, and the results obtained follow.

1. Levaditi C strain of herpes virus in the brain, and 2 days later neurovaccine virus in the cornea. Typical neurovaccine virus keratoconjunctivitis resulted, from which the animals recovered without cerebral involvement.

2. Levaditi herpes virus in the brain, and 2 days later neurovaccine virus in the shaved abdominal skin. There followed no encephalitis, only neurovaccine dermal lesions, from which the animals promptly recovered.

3. Levaditi herpes virus in the brain, and 2 days later neurovaccine virus in the testes. Orchitis developed but no encephalitis.

4. Levaditi herpes virus in the brain, and at the same time neurovaccine virus also in the brain. Since both viruses are inactive in guinea pigs by this method of injection, but produce characteristic encephalitis in rabbits after intracerebral inoculation, guinea pigs were subjected to inoculation simultaneously as follows: (a) the usual amount (0.15 cc.) of both viruses was injected separately in each hemisphere, and in another series larger amounts (0.2 cc.) were tested; (b) usual quantities (0.15 cc.) of the two viruses mixed *in vitro* were introduced into one hemisphere and in another test larger doses (0.2 cc.) were employed. None of these animals developed encephalitis.

It was not found possible, therefore, to implant the Levaditi virus on the cerebrum of guinea pigs by combining it with neurovaccine virus in such a way as to permit both to act with maximum effects at the same time.

Cross-Immunity in Guinea Pigs. Levaditi and H.F. Strains.

Cross-immunity in rabbits between the Levaditi and the H.F. strains of herpes virus has been determined by Flexner and Amoss.[17] The tests to be reported relate to the corresponding effects in the guinea pig.

Three series of experiments were made at different times, each series containing 3, 6, and 3 guinea pigs immunized with Levaditi herpes virus. In addition, guinea pigs and rabbits were included to serve as controls of the activity not only of the Levaditi virus used in the immunization, but also of the H.F. strain given as a test injection later.

The procedure of immunization consisted in injecting guinea pigs intraperitoneally with 2 cc. of a 25 per cent saline suspension of fresh rabbit brain containing active Levaditi virus. Four to five such inoculations were made at 4 to 7 day intervals. From 7 to 10 days after the last injection, the guinea pigs were given intracranially 0.2 cc. of a 10 to 20 per cent suspension of either guinea pig or rabbit fresh brain containing the H.F. strain of herpes virus. The control animals showed characteristic herpes-virus encephalitis with death in 6 to 7 days, while the immunized guinea pigs remained well.

Cross-immunity has therefore been shown to exist in the guinea pig between the Levaditi virus and the H.F. strain of herpes virus. Moreover, by repeated intraperitoneal injections of the Levaditi virus, during which no apparent signs of disease were noted, a solid immunity was produced.

[17] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 357.

DISCUSSION AND SUMMARY.

A number of methods have been employed in attempts to induce encephalitis in guinea pigs with the Levaditi C strain of herpes virus. Some of these consisted of different modes of inoculation of the virus itself and others of different ways of combining it with vesicular stomatitis and neurovaccine viruses so as to obtain the concomitant effects of both. In still another test the Levaditi virus was combined with the neurovaccine in a manner calculated to bring about the maximum action of each at the same time. By all these methods, the Levaditi virus failed to evoke the characteristic encephalitis which this specimen is capable of inducing uniformly in rabbits. On the other hand, when the Levaditi herpes virus is inoculated into the brain of guinea pigs in conjunction with suitably timed corneal injections, it acquires active encephalitogenic properties.

The results just noted suggest several considerations:

1. The possibility of increasing the virulence of a filtrable virus by animal passage in a special manner. It is not likely that the increase as observed was due to dosage, for after the virus acquired its encephalitogenic property for guinea pigs, the usual amounts of virus suspensions sufficed to induce, in a uniform way, typical encephalitis.

2. The opinion previously expressed by Flexner[2] that the guinea pig serves merely to separate weak from strong strains of herpes virus is supported: for only according to the particular method described, could the encephalitogenic power of the Levaditi virus be developed and the weak be converted into a strong herpes strain. With the acquisition of this power, the Levaditi virus acted in precisely the same manner as strong herpes strains both in the guinea pig and the rabbit. Moreover, it was shown in guinea pigs that cross-immunity occurs between weak and strong strains.

3. The two samples of neurovaccine virus employed were incapable of inducing encephalitis in guinea pigs after intracutaneous, intratesticular, corneal, or intracerebral inoculation, although they were actively encephalitogenic in rabbits. In spite of the fact that the vaccine virus and herpes virus are different, as shown by the histopathology and absence of cross-immunity, they behave in the same way when injected into the brain of the guinea pig. The failure of the concomi-

tant action of both viruses to induce encephalitis in the guinea pig suggests that the association of two viruses, under the experimental conditions outlined, is incapable of inducing encephalitis, if either, by itself, is non-encephalitogenic.

4. The serum from normal guinea pigs may neutralize a weak (Levaditi C) but not a strong (H.F.) strain of herpes virus; but the neutralizing action of the serum on Levaditi C virus is not uniform.

5. The Levaditi strain of virus can increase in quantity in the brain of the guinea pig to a degree which permits detection and yet fails to evoke any distinctive clinical picture or definite histopathological changes.

6. Repeated intraperitoneal injections of Levaditi virus in guinea pigs elicit no signs of infection, yet they induce a solid immunity to strong strains of herpes virus.

STUDIES ON INDIFFERENT STREPTOCOCCI.

I. Separation of a Serological Group—Type I.

By C. H. HITCHCOCK, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In the search for the etiological agent of rheumatic fever, attention has frequently been called to the possibility that streptococci may in some way be concerned in the production of the disease. Mostly the suggestions have dealt with organisms of the *viridans* group, less frequently with the hemolytic group, but only recently has it been proposed by Small (1) and Birkhaug (2) that the indifferent streptococci must be considered in this connection. This newer view-point is explainable when it is remembered that the bacteria isolated during life or at necropsy from patients with the disease have usually been *Streptococcus viridans*, and that the demonstration of the indifferent varieties has been a much less common occurrence.

The existence of the indifferent streptococci has been a matter of common knowledge for some decades.

Mandelbaum (3), who mentioned their occurrence in the throat and elsewhere, regarded them as simply a variety of saprophytic organism. Zangemeister (4), commenting upon their lack of virulence, considered them apparently as a degraded form of *S. hæmolyticus*, and believed that under proper conditions certain of them could assume the properties of the latter organism. Their cultural characteristics were dealt with by Brown (5), and a tentative classification upon the basis of certain fermentation reactions was advanced.

That the indifferent streptococci were not completely apathogenic was suggested by the observations of Kinsella (6), who recovered them in four instances from the blood stream of patients suffering from subacute bacterial endocarditis. Furthermore, Rosenow (7) on a few occasions isolated them from joint punctates of acute rheumatic fever patients. Working with a strain grown from the blood of a patient suffering from the latter disease, Small (1) demonstrated that inoculation of this organism intravenously into rabbits resulted in the production of lesions which strikingly resembled those described by Bracht and Wächter (8)

and by Thalhimer and Rothschild (9) and Cecil (10), and others following similar injections of *S. viridans*. Treatment of patients with an antiserum prepared with this strain produced effects recalling those reported two decades ago by Menzer (11) and others as resulting from the treatment of acute rheumatic fever patients with polyvalent antistreptococcus serum. More recently Birkhaug (2), Kaiser (12), and later Swift, Wilson, and Todd (13), have called attention to the fact that certain strains of indifferent streptococci possessing in common the property of fermenting inulin yield culture filtrates which on intradermal injection in moderate dilution produce reactions in individuals suffering from rheumatic fever as well as from other conditions. Perhaps the most striking observation in this connection is the fact that heating this filtrate in boiling water for 1 hour augments its "toxicity" (13). Birkhaug has also suggested that his inulin-fermenting organisms show a certain degree of serological interrelationship.

In order for different workers in a field such as this to be able to compare results satisfactorily, some sort of classification of the organisms in question is desirable. Despite the difficulties encountered by others in classifying streptococci of the hemolytic and *viridans* types, it seemed of interest to examine a number of strains of indifferent streptococci, and to determine whether any simple serological grouping was possible. For this purpose 159 strains were secured. Sixteen of these were obtained through the kindness of Dr. Birkhaug, including the R F 1 strain with which much of his work was carried out. These were all inulin-fermenting organisms of the type described in his paper. Later, as the work was in progress, Dr. Small kindly forwarded two cultures, R 1 and R 9; the former was that strain originally isolated and described by him (1). The remaining 141 were secured from the throats of patients suffering from various diseases, including rheumatic fever, from the throats of normal individuals, or from the interior of tonsils removed at operation. Two of our strains were isolated from blood cultures of patients with acute rheumatic fever.

Methods.

Culture material was obtained from the throats with sterile swabs and from the interior of excised tonsils after sterilization of the surface, and the initial seeding was made upon plain blood agar plates. After an incubation period of 24 to 36 hours colonies of indifferent streptococci were transferred to plates of Birkhaug's medium (2) from which the bile was omitted. These plates served as a rough means for the separation of the inulin-fermenting and non-fermenting types. From these plates strains of both varieties were chosen, and subsequently were

kept in stock culture in blood broth. Final determinations of the capacity of the organisms to ferment inulin and salicin were carried out with tubes of Hiss serum water. Incubation was prolonged for 10 days before negative results were recorded.

Antisera were prepared by the intravenous inoculation into rabbits of whole 24-hour cultures in phosphate broth pH 7.8 containing 0.05 per cent of dextrose. The injections were given semiweekly over a period of 6 to 8 weeks. During the first 2 weeks amounts of 0.5 cc. and 1 cc. respectively of heat-killed culture were administered. Thereafter living culture was employed in doses of 0.5 cc., 1.0 cc., and finally of 2.0 cc. The latter dose was repeated in case trial bleedings failed to reveal a satisfactory titer of agglutinin. The animals were exsanguinated 8 or 9 days following the last dose. By this means it was a simple matter to obtain sera which would agglutinate the homologous organisms in dilutions of 1:5000.

For carrying out agglutinations, the bacteria were grown for 24 hours in phosphate broth pH 7.8 containing 0.05 per cent dextrose. Usually the resulting culture formed a homogeneous, turbid suspension, which for the purpose in hand was extremely satisfactory. In a few instances concentration with the centrifuge was necessary to obtain a sufficiently dense suspension; and occasionally growth was so granular as to render the culture useless for agglutination. Antisera were diluted 1:125, 1:250, 1:500, and 1:1000 in phosphate broth; 0.5 cc. of each of these dilutions was mixed in small tubes with 0.5 cc. of bacterial culture; all tests were recorded in terms of the final dilution of serum which resulted. On each test day a series of similar dilutions of normal rabbit serum was employed with each strain under investigation. Suspension controls were set up, in which 0.5 cc. of culture was mixed with 0.5 cc. of phosphate broth. All tubes were kept in an incubator at 56° for 3 hours, at the end of which time readings were recorded.

Antigens for use in the precipitation test were prepared as follows: 35 cc. of a 24-hour growth in 1 per cent dextrose broth were centrifuged, and the sedimented organisms were suspended in 10 cc. of N/100 NaOH in physiological salt solution. In a few instances in which growth was light, as estimated roughly from the volume of the packed organisms, smaller amounts of the alkaline solution were used. These suspensions were permitted to stand overnight in the ice box, at the end of which time they were centrifuged. The clear supernatant fluid was acidified with 10 per cent acetic acid, whereupon a rich precipitate of acid-insoluble protein appeared. This was permitted to flocculate overnight in the ice box, and was then centrifuged off. The water-clear supernatant fluid was neutralized with N/1 NaOH, and was then ready for use. In carrying out the tests 0.1 cc. of antiserum was placed into each of a series of small tubes, and the antigen was added in amounts of 0.2, 0.1, and 0.025 cc.; sufficient normal salt solution was added to make the final volume 0.5 cc. A control series was always employed in which the same amounts of antigen and salt solution were added to 0.1 cc. of normal rabbit serum. The tubes were kept in the water bath at 37°C. for 2 hours, following which they were placed in the ice box; readings were made the following

morning.   The resulting precipitates were found to have gathered in the bottom of the tubes in the shape of coherent discs, which strongly resembled those produced when pneumococcus specific soluble substance is mixed with its corresponding antiserum; this suggested the possibility that substances of similar nature were extracted by this simple procedure from the indifferent streptococci.

Parallel agglutination and precipitation tests were carried out as above outlined with each of the 159 strains.   At first there were employed four sera, prepared respectively with two of Dr. Birkhaug's strains, and two of our own.   Inasmuch as it was soon found that interchangeable results were obtained with all four sera, it was considered permissible to perform all of the agglutination tests with a single serum (R 867), and all of the precipitations with another (R 862).   These sera were of approximately the same titer.   The first was prepared against Strain Q 88 E, which was isolated from the blood stream of a patient with rheumatic fever.   The second was prepared against Strain RF 24 T, secured from Dr. Birkhaug and originally isolated by him from the throat of a patient with the same disease.   Results were recorded in the usual way with $+$ signs, $+ + + +$ being the maximum; and except for a few instances which will be considered below the results of the two tests were found to check rather closely.

RESULTS.

In Table I are given in summary the results of the observations upon these 159 strains.   In the group designated "Inulin fermentation prompt," acid and clot were formed in the Hiss serum water within a period of 48 hours.   The group designated "Inulin fermentation delayed" required longer periods of time, and occasionally clot formation remained in abeyance.

As the result of these observations it is possible to separate the strains into two large groups.   The first consists of those organisms which show strong agglutination and precipitation reactions with the sera employed.   This serological uniformity seems sufficient warrant for the inclusion of all these strains within a single category, which may be designated as Type I.   As might be expected, the organisms are not absolutely identical, as revealed by minor variations in the strength of the reactions.   Included under Type I are a few strains which fail to conform exactly to the above conditions: (1) some which show spontaneous agglutination, but which can be typed by means of the precipitin reaction, (2) others which agglutinate to titer in the Type I serum, but from which it is not possible to extract the precipitating antigen in adequate concentration by the standard method.   In the

latter case it may be tentatively assumed either that in a few strains the antigen is present within the bacterial cell in materially smaller amounts than usual, or that, inasmuch as the antigens from different strains do not of necessity possess the same degree of stability, an

TABLE I.

	Inulin fermentation	Agglutination Serum R 867	Precipitation Serum R 862	Strains
Type I	Prompt	++++	++++	45
	"	++++	+++	11
	"	+++	++++	5
	"	+++	++ to +++	5
	"	Spontaneous	+++ to ++++	5
	"	++++	+ to ++	9
	"	+++	+	1
	"	++++	− to ±	2
	"	+++	− to ±	3
Total..				86
Group X	Prompt	++	− to ±	2
	"	±	++	1
	"	± to +	± to +	7
	"	− to ±	− to ±	3
	"	Spontaneous	+ to ++	4
	"	"	− to ±	15
	Delayed	++	+	1
	"	+	+	2
	"	+	±	1
	"	−	−	3
	"	Spontaneous	+	1
	"	"	− to ±	8
	Negative	+	± or −	5
	"	−	± or −	14
	"	Spontaneous	± or −	6
Total..				73

occasional one may be injured by the reagents employed. All the strains of Type I ferment both inulin and salicin promptly.

The second group comprises those organisms which agglutinate weakly or not at all in Type I antiserum at a dilution of 1:250 or higher, and which fail to yield strongly precipitating extracts by the

TABLE II.

Class	Strain	Inulin	Salicin	Agglutination: Serum R 867 Serum dilutions				Precipitation: Serum R 862 Antigen in cc.		
				1:250	1:500	1:1000	1:2000	0.2	0.1	0.025
Type I	Q 88 E	+ prompt	+	++++	++++	+++	++	+++±	++++	++
	RF 24 T	"	+	++++	++++	+++	++	+++±	+++	++
	Q 166 B	"	+	++++	++++	+++	+	+++±	(±)	+±
	P 5 B	"	+	++++	++++	++++		±+	(±)	–
	P 86 B	+	+	Spontaneous		++++±	++	++++	++++	+++
Group X	RF 156	+ prompt	+	++	+	–	–	±+	(±)	–
	R 9	+	+	++	+	–	–	?	±	–
	P 28 B	+ (4 days)	+	–	Spontaneous	Spontaneous		?	(±)	–
	P 71 B	–	+		–	–	–	±+	(±)	–
	P 128	–	+	–	Spontaneous	Spontaneous		?	–	–
	R 1	–	+		–	–	–	±+		–
	P 16 B	–	–		–	–	–	–	–	–

standard method employed. In general the same correspondence appears between the results of the agglutination and precipitation tests. It is recognized that in still lower dilutions of the serum agglutination might occur, and in a few instances this possibility has been tested and found to be a fact, but such slight cross reactions in a high titer serum are not of significance from the point of view of the present study. A greater percentage of the strains in this group show a tendency to flocculate spontaneously than is the case with those of Type I. The differentiation of the strains of this group from those of Type I is distinct, but the data are insufficient to warrant the delimitation of further types at present. These strains may, however, be placed together provisionally as Group X, with the understanding that this is a frankly heterogeneous collection from which in the future it may well prove possible to separate further definite types. The members of this group may vary in their capacity to ferment inulin: in some it is well marked; in others it becomes apparent only after a lapse of several days; while in the case of many strains it is entirely lacking. The group has been further studied from the point of view of salicin fermentation. Slight precipitation occasionally occurred with the Type I serum when extracts from salicin-fermenting organisms were employed, but was never observed in the case of non-salicin fermenters. In view of the fact that all of Type I strains are salicin fermenters this cross relationship is noteworthy and suggests that the strains which do not ferment salicin are the least closely related to Type I of any studied.

In Table II is presented a composite protocol, which serves to illustrate the manner in which the data were collected and the grouping of the individual strains arrived at. It is possible that certain of the weak precipitin reactions recorded in Group X were traceable to small protein residues in the bacterial extracts. Inasmuch as only the acid-insoluble protein was removed, the solutions were obviously far from containing one antigen only.

It should be noted that in our hands Strain R 1 has consistently failed to ferment inulin unless adapted following six to eight transfers in inulin serum water, or unless faulty inulin was employed. In Small's original report this strain is designated as an inulin fermenter. This divergence may be explainable by differences in the media used.

Unless great care is observed in sterilization the inulin will be fermented by many ordinary non-fermenters.   Certain lots of inulin are also easily fermented by most indifferent streptococci.   It is, therefore, necessary to control carefully each new lot of media.   In this work if a strain failed to ferment salicin but fermented inulin the inulin was always found to be at fault.

DISCUSSION.

The existence of a group of indifferent streptococci sufficiently compact to warrant separation as a distinct type suggests certain analogies to conditions within the pneumococcus family.   As far as the present observations indicate, the antigen complex of these streptococci contains a substance which by the analogy of disc precipitation in immune serum may be regarded as comparable to the pneumococcus specific substance.   This analogy is strengthened by the fact that the active substance is destroyed by boiling for 30 minutes with $N/10$ HCl, and that as this hydrolysis proceeds copper-reducing substances appear in the solution.   The usually close agreement between the results of the agglutination and precipitation reactions suggests that it is this same substance which is responsible for both of the immune reactions, and that the compactness of Type I results from the presence within a great number of strains of indifferent streptococci of soluble substances identical or very similar chemically. Such a concept is comparable to that developed by Avery and Heidelberger (14) in the case of the pneumococci, and by Lancefield (15) for certain strains of *Streptococcus viridans*, but is at variance with conditions as they exist within the family of the hemolytic streptococci (16, 17).   Efforts to identify the soluble substances of Type I with the species-specific soluble substance of the hemolytic streptococci have been unsuccessful.   On the other hand, a few strains of *Streptococcus viridans* have been encountered which have shown agglutination in Type I serum, and from these strains it has been possible to prepare extracts which precipitate such serum with the formation of fairly compact discs.   Such strains of *Streptococcus viridans*, therefore, contain antigens in common with Type I indifferent streptococci. Similar instances of cross agglutination have been reported by Birkhaug.   Whether such observations have more than an academic interest is at present a matter of conjecture.

SUMMARY AND CONCLUSIONS.

Serological study of a large number of strains of indifferent streptococci has revealed the existence of a large homogeneous group to which the designation Type I has been applied. It is recognized that members of Type I are not necessarily identical, and that further division into subtypes may be feasible. All strains of Type I ferment inulin and salicin.

The remaining strains are referred to as belonging to Group X. They are distinguished only by their failure to react strongly with Type I serum. While at present this group must be regarded as quite heterogeneous, further work may reveal the presence of other as yet undefined types now included within its limits. The organisms of this group vary in their fermentative reactions with both inulin and salicin.

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STUDIES ON INDIFFERENT STREPTOCOCCI.

II. Observations on the Distribution of Indifferent Strepto- cocci in the Throats of Rheumatic and Non-Rheumatic Individuals.

By C. H. HITCHCOCK, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Within the past year the attention of investigators in the field of rheumatic fever has been sharply directed to the indifferent strepto- cocci by the work of Small (1) and of Birkhaug (2). The former has cultivated organisms of this type from patients with the disease, and has prepared a therapeutic serum with which results are reported similar to those obtained with other varieties of antistreptococcus sera (3). He has assumed that these bacteria are to be regarded as the specific causative agents of the disease. Birkhaug has recovered streptococci of the same type from blood, throats, tonsils, and feces of patients suffering from the disease, and with culture filtrates of these organisms has obtained a high percentage of positive skin reac- tions in individuals "stigmatized by rheumatic fever or its syndromes." Neither of these investigators has adduced convincing evidence that indifferent streptococci are to be more seriously considered in connec- tion with this disease than are streptococci of the *viridans* type.

It might be thought that, if the indifferent streptococci played a special rôle in the production of rheumatic fever, they should be par- ticularly abundant in the throats of patients during the course of the disease or during early convalescence. If such were the case, they should be found with especial ease during the period of acute tonsil- litis or pharyngitis which usually constitutes the initial stage of the illness and is frequently observable at the time of hospitalization. It seemed, therefore, of interest to study the flora of the throats of a group of patients suffering from one or another of the acute manifesta- tions of rheumatic fever. As control groups there were studied (1)

patients who had recovered from the disease, symptom-free for at least several months and usually for a year or more; (2) patients suffering from other conditions; (3) normal individuals. The persons constituting the last two groups were free from any condition suggesting the rheumatic syndrome, and with one or two exceptions had never suffered from any of its manifestations.

Methods.

Cultures were obtained from throats by simple swabbing of the tonsils, or tonsillar fossæ, and pharynx. Initial seedings were made upon blood agar plates, spread radially with a platinum loop. At first plates of the inulin-bile-serum agar medium described by Birkhaug (2) were also employed, but these were found to present no unusual advantages and were soon abandoned for purposes of isolation. After an incubation period of from 24 to 36 hours the plates were studied, and a rough estimate was made of the percentage of indifferent colonies present. From six to eight colonies were then picked to blood agar plates, and after appropriate incubation smears were examined microscopically. All strains of indifferent streptococci were then transferred to plates of 1 per cent inulin agar enriched with chest fluid, and at the end of 48 hours the relative numbers of inulin-fermenting and non-fermenting strains were recorded. This method served roughly for initial differentiation, although with many strains it was found that inulin fermentation proceeded readily in tubes of Hiss serum water though it had failed to become evident when the plate method was employed. From the inulin agar plates strains were transferred to tubes of blood broth which were preserved in the ice box for further study. Usually two strains from each culture were thus preserved.

Final determinations of the capacity of the organisms to ferment inulin and salicin were made with tubes of Hiss serum water containing the appropriate carbohydrate. Inocula consisted of 0.2 cc. of a 24-hour culture in broth containing 0.05 per cent of dextrose. No result was recorded as negative until a 10-day period of incubation had elapsed. Two strains from each culture were examined serologically according to methods described previously (4). In most instances both agglutination and precipitation reactions were employed; after the close correspondence between the results of these two methods had been established, it was considered permissible in the case of a few strains to employ the reaction of agglutination alone.

RESULTS.

In Table I are presented data based upon the total number of cultures studied, analyzed with reference to the occurrence of indifferent streptococci.

Regardless of the presence or absence of rheumatic fever, there seems to be a tendency for these organisms to be found somewhat more frequently in the throats of individuals suffering from some condition of

TABLE I.

Incidence of Indifferent Streptococci in Throat Cultures.

	Total cultures	Occurrence of indifferent streptococci	
		Present	Absent
Active R.F. or early convalescence..........	34	29 (85%)	5 (15%)
R.F. recovered............................	26	24 (92%)	2 (8%)
Other conditions.........................	36	32 (89%)	4 (11%)
Normal individuals.......................	49	37 (76%)	12 (24%)
	145	122(84%)	23(16%)

TABLE II.

Incidence of Indifferent Streptococci in Throats of Individuals.

	Total persons	Occurrence of indifferent streptococci	
		Present	Absent
Active R.F. or early convalescence..........	28	26 (94%)	2 (6%)
R.F. recovered............................	23	21 (91%)	2 (9%)
Other conditions.........................	36	32 (89%)	4 (11%)
Normal individuals.......................	45	36 (80%)	9 (20%)
	132	115 (87%)	17 (13%)

disease than in those of normal persons. The differences are not, however, very striking. It is noteworthy that the bacteria under consideration were harbored quite as frequently by patients free from any taint of rheumatism as by those who were definitely suffering from

some manifestation of the disease.   Certainly it would appear justi-
fiable to conclude that the indifferent streptococci are widely distrib-
uted, carried more or less constantly by roughly 85 per cent of the
population, and that there is a slight and entirely non-specific tend-
ency for them to be found more frequently in the throats of hospital
patients than in those of normal individuals.   In view of the innate
inexactness of the method, the results in the various groups are sur-
prisingly concordant.   Inasmuch as in a few cases more than one
culture was made from a single individual, and as when indifferent
streptococci are scarce they may be missed in the first culture and
be found in the second, or *vice versa*, it has seemed desirable to present

TABLE III.

Percentage Incidence of Indifferent Streptococci in Throat Cultures.

	Total cultures	1–5%*	6–15%	16–30%	Above 30%	Present**
Active R.F. or early convalescence...	34	17 (50%)	5 (15%)	1 (3%)		6 (18%)
R.F. recovered.....................	26	16 (62%)	3 (11%)	3 (11%)	1 (4%)	1 (4%)
Other conditions....................	36	22 (61%)	4 (11%)	3 (8%)	2 (6%)	1 (3%)
Normal individuals.................	49	22 (45%)	9 (18%)	5 (10%)		1 (2%)

*Includes a few early cultures described as containing "few" or "very few."
**Includes a few of the first cultures in which estimates were not made.

Table II, in which the data are analyzed in terms of individuals rather
than of total cultures.

Comparison with Table I shows that there is a tendency for the
percentage of negative results to decrease when repeated cultures are
taken from the same group of individuals.   In all probability every
person now recorded as free from these streptococci would eventually
be found to harbor them, though in very small number, if the cultural
procedure were repeated with sufficient frequency.

Although the indifferent streptococci were found no more frequently
in the throats of one group of individuals than in those of another, it
might be considered that in the case of patients suffering from some
active manifestation of rheumatic fever these bacteria would consti-

tute a much greater percentage of the total flora than would be the case with other individuals. In Table III are presented data which bear upon this point.

The cultures from each group of individuals have been divided into subgroups, according as the indifferent streptococci constituted 1–5 per cent, 6–15 per cent, 16–30 per cent, or more than 30 per cent of the total flora, and the total number of cultures in each subgroup have been tabulated, together with the percentage of the main group formed by each subgroup. That the percentages under each group do not add up to 100 per cent results from the omission of a column for the negative cultures.

TABLE IV.

Incidence of Groups of Indifferent Streptococci in Throats of Individuals.

	Total strains	Distribution into groups	
		Type I	Group X
Active R.F. or early convalescence..........	43	21 (49%)	22 (51%)
R.F. recovered............................	32	13 (40%)	19 (60%)
Other conditions.........................	43	20 (46%)	23 (54%)
Normal individuals.......................	56	30 (53%)	26 (47%)

In slightly more than half of the individuals indifferent streptococci were found to constitute 1–5 per cent of the total throat flora, irrespective of the presence or absence of rheumatic fever or its stigmata. Thus, in 45 per cent of all the cultures from normal individuals these organisms were estimated as forming 1–5 per cent of the total flora. Reference to the remainder of the table shows that there was no tendency for these streptococci to occur in greater abundance in the throats of patients with rheumatic fever than those of other patients. In fact, some of the highest percentages recorded were found in persons free from any taint of rheumatism, while, conversely, it was several times observed that during very acute relapses of this disease there was a tendency for the plates to be crowded with *Streptococcus viridans*, largely to the exclusion of other types of organism.

In Table IV the total number of strains studied from each group of individuals is analyzed with respect to their serological characteristics (4).

In view of the fact that only one or two strains from each positive culture were studied, so that the element of chance played a large part, there is a remarkable agreement between the percentages for the various groups of persons. The probability that an indifferent streptococcus chosen at random from a throat culture will belong to Type I or will fall into the heterogeneous Group X is apparently independent of the condition of health of the individual from whom the culture is taken. In other words, there is no greater tendency for the organisms of Type I to be found more frequently in the throats of one group of individuals than in those of another, and in rheumatic fever there is no tendency for the incidence of this type to vary in either direction.

DISCUSSION.

The isolation of organisms of various types from lesions of rheumatic fever has been a more or less frequent occurrence. Many of the reports are open to grave suspicion of error in technique or of interpretation. The best accredited results are those in which streptococci of various types have been found. Never, however, has it been possible to secure experimental evidence that these organisms are directly concerned in the production of the disease. Their injection into laboratory animals has never been followed by the development of lesions closely resembling those of rheumatism. The pathological findings in the joints have been usually those of a proliferative and destructive arthritis (5, 6) while in the heart vegetative endocarditis has been present, together with the Bracht-Wächter bodies (7) in the myocardium. This has been the case regardless of whether the streptococci were of the indifferent or *viridans* type. Recent investigations (8, 9) have suggested that the failure of all these attempts may not mean that the bacterium used was unassociated with rheumatic fever, but that the error has been one of method. That is, the preliminary induction of the necessary tissue allergy in the experimental animal may be the *sine qua non* which hitherto has been neglected. Given the proper condition of tissue sensitization any streptococcus possessed

of the requisite allergizing capacity may be able to give rise to the peculiar condition called rheumatism.

The most recent organism to be ascribed a specific etiological rôle in rheumatic fever has been the indifferent streptococcus. The first object of this present investigation was to determine the extent of the distribution of these organisms among selected samples of the population and the percentage of the total flora of the throat which they formed. In spite of the fact that bacteriologists have been familiar with these organisms for decades, no such study has to our knowledge ever been carried out. With these fundamental facts established, it was then proposed to compare the flora of the throats of a number of groups of individuals, to determine whether in the case of patients suffering from rheumatic fever they occurred in greater incidence or in higher percentage. Such was not the case. There was found a slightly higher incidence of indifferent streptococci in the throats of hospital patients than in the throats of normal persons; but from the present study it is apparent that these bacteria are no more widely distributed among patients suffering from rheumatic fever than among other groups of patients. In the majority of throats they are present in approximately the same percentage relative to the total flora. Brown (10) has observed that in inflammatory conditions of the throat they tend to be overgrown by other organisms, notably the hemolytic streptococcus; and in a few cases of acute rheumatic fever included in the above tables there has been a tendency for *Streptococcus viridans* to displace them in similar fashion. Certainly such findings are not in harmony with the claim that these organisms are the sole cause of the disease.

Future investigation will probably determine the rôle which the indifferent streptococci actually play in the disease. Doubtless they must be considered along with the other types of streptococci in any comprehensive theory of its etiology. That they are not simple saprophytes is suggested by the facts that occasionally they may be the cause of bacterial endocarditis (11), that they are often efficient allergizing agents (12), and that they possess the capacity of yielding "toxic" filtrates (2, 13, 14). Therein they do not differ from many strains of *Streptococcus viridans*, and consequently are no more entitled to special consideration than are the latter.

It may be objected that the cultural and serological types here reported are not exclusively those used by Small (1) in the preparation of his SCA antiserum, and hence the validity of these conclusions may be questioned.   In this connection attention should be directed to the fact that among the strains investigated in these two studies there were RF 1 recovered by Birkhaug from blood cultures of a rheumatic fever patient and Q 88 E and Q 97 EA similarly recovered by us from patients with the active disease.   All of these fell into Type I.   The R 1 strain and R 9 strain recovered by Small differ from each other and are also heterogeneous to Type I.   While these strains are closely related as judged from their effect on blood agar, still their serological heterogeneity casts the same doubt on their specific etiologic rôle as was cast on *Streptococcus viridans* and for the same reason.   We feel, therefore, that indifferent streptococci play a rôle in the production of this disease similar to that exerted by the green-forming streptococci.

SUMMARY AND CONCLUSIONS.

Indifferent streptococci occur in comparatively the same abundance in the throats of patients suffering from rheumatic fever or early in convalescence from the disease as they do in those who have recovered from the disease, or in those of patients suffering from other diseases.

There is a slightly increased incidence of these microorganisms in the throats of hospital patients as compared with those of normal individuals.

Type I occurs with comparatively equal frequency and abundance in the throats of all four classes of individuals studied.

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FURTHER EXPERIMENTS WITH THE INTRADERMAL PNEUMOCOCCUS INFECTION IN RABBITS.

By KENNETH GOODNER.

(From the Department of Bacteriology and Immunology of Harvard University Medical School, Boston.)

In a previous communication (1) we have described the characteristic symptom-complex brought about by infecting rabbits intradermally with Type I pneumococci. The following paper is a continuation of that report.

I.

Specific Serum Therapy.

Since we believe that the rabbit experiment as described in our first paper promises a method of determining the therapeutic potency of specific antipneumococcic serum more satisfactorily than the now prevalent mouse protection technic we have given considerable attention to the effects of serum administration on the course of the pneumococcus infection of rabbits, both in regard to the local and general phenomena.

The following points have already been established:

1. That animals recover promptly after the intravenous administration of large amounts of antipneumococcic serum.

2. That there are four important features of this induced recovery, as follow: (*a*) the immediate and permanent disappearance of organisms from the circulating blood; (*b*) a drop in temperature to normal levels within 24 hours; (*c*) the disappearance of organisms from the local lesion in the course of 4 to 20 hours; (*d*) loss of inflammatory color of the local lesion within 24 hours, usually accompanied by epidermal desquamation.

3. That for a given serum at a given stage of the disease there may be established a *minimal effective dosage* (M.E.D.) such that larger

amounts give no added therapeutic value and smaller amounts are insufficient to bring about recovery.

Minimal Effective Dosage at Various Stages of the Disease.—It is a common clinical observation that the amount of serum which might be used effectively on the 1st or 2nd day of lobar pneumonia is insufficient if given at a later time. Some clinicians have even stated that after the infection has progressed beyond a certain point no amount of serum will save.

The question of a quantitative element has been studied in the experimental "dermal pneumonia" of the rabbit by determining the minimal effective dose of a given serum for each of three stages of the disease. It is necessary to stress again the sharpness of the end-point in determinations of the effectiveness of therapeutic sera. The results of such an experiment are shown in Fig. 1.

This experiment shows that the amount of serum necessary for effective therapy increases very rapidly as the disease progresses and emphasizes the importance of early treatment. Even the time occupied in the ordinary typing process would greatly lessen or entirely eliminate the chances of successful therapy.

For this particular serum, if one assumed a definite relationship between therapeutic dose and body weight of the animal and transferred these proportions to man, the results would be as follow: In the standard rabbit (1500 gm.) we have found it necessary to use serum to the amount of 0.23 per cent of body weight at 24 hours, 0.47 per cent at 48 hours, and approximately 0.67 per cent at 72 hours. This would indicate that in a man of 70 kilos it would be necessary to use 150 cc. of this serum at 24 hours, 325 cc. at 48 hours, and approximately 475 cc. at 72 hours.

In the experimental rabbit condition it is not essential that the minimal effective level be exceeded at a single injection but if multiple injections are used these must not be too far apart and the total quantity must exceed the minimal effective amount for that period. Unless this effective level is exceeded at a given time absolutely no beneficial result has been obtained.

Bloomfield (2) advances the opinion that in lobar pneumonia the usefulness of serum depends upon the presence and seriousness of the bacteriemia. The rabbits which we have studied have shown some

variation in the severity of the bacteriemia at the time of treatment but the minimal effective dosage of the serum appears to be a fixed quantity for a given period and this does not vary with the severity of the bacteriemia.   If such a correlation did exist it might be anticipated that ten times as much serum would be necessary at 48 hours
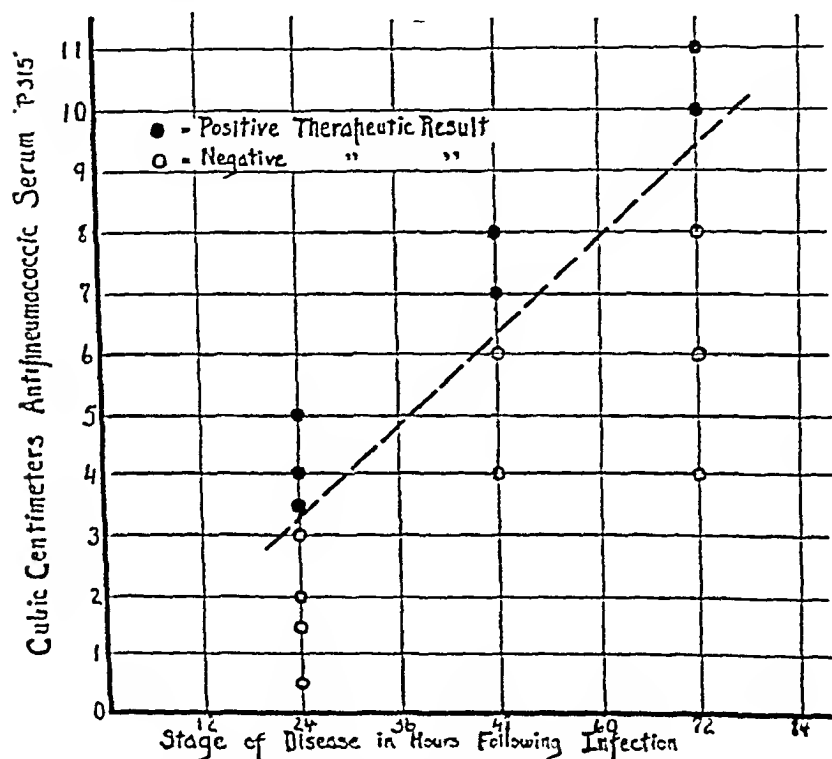
Fig. 1. Determinations of the minimal effective dosage of Antipneumococcic Serum P 315 at various stages of the disease.   The diagonal line represents the suggested effective level of therapy.

as was necessary at 24 hours, for the number of bacteria per cc. of blood has often increased by a multiple of 10 or even of 100.

The contour of the suggested boundary curve, shown by the broken line in Fig. 1, suggests that there is a progressive rise in some element which unites quantitatively with the antiserum or quantitatively

antagonizes it. Proponents of the pneumonia toxin theory have advanced the idea that there may be a progressive accumulation of some toxic element in the tissues.

Decision as to the significance of the findings presented in Fig. 1 must await the solution of this question. Our results have shown, however, that there is an experimental basis for early and vigorous treatment of lobar pneumonia, and would seem to point to the immediate use of some sort of polyvalent serum, since the time required for typing greatly decreases chances for success with practicable amounts of serum.

The use of agglutinin titer or protective value of a specifically treated patient's serum in guiding further specific therapy has been suggested by Sutliff (3). The qualitative detection of agglutinins and protective substances in the treated rabbit indicates only that it has been treated; even if such tests are made quantitatively the information gives only the approximate dilution that the therapeutic serum has undergone with the rabbit's own serum. It is obvious that qualitative tests have little value since various antibody levels are necessary at various stages of the disease in order to bring about successful therapy.

The Comparison and Standardization of Antipneumococcic Sera on the Basis of Therapeutic Value.—The earlier workers used agglutinin titer as a basis for comparison of probable therapeutic efficacy of sera. This method has given way to a titration in which mouse protection is the indicator of protective value. The Hygienic Laboratory has established a certain standard and furnishes for comparative purposes a serum which fulfills this requirement. This standard is not based on any known quantitative therapeutic value for cases of lobar pneumonia.

Having found the minimal effective dosage at a given hour in a 1500 gm. rabbit to be a characteristic of the serum and of a constant value for that serum, experiments were planned by which this might be made the basis for the comparison of sera.

Seven sera were selected for comparison. The general plan of work consisted in determining for each of the sera the mouse-protective value, the agglutinin titer, and the therapeutic minimal effective dosage for rabbits at 24 hours.

The great interest in this table lies in the fact that the mouse-protective doses listed in the column of mouse-protective values show the extraordinary potency of the serum in protecting a highly susceptible animal when serum and organisms are mixed before injection, an experimental condition which is comparable in absolutely no respect to the condition under which the serum is therapeutically used. In the second column under "Rabbit therapeutic value," also calculated for 1 cc., is shown the much diminished potency, though still considerable, when the serum is used in another highly susceptible animal under conditions which, we believe we are justified in assuming, simulate

TABLE I.

Comparison of Agglutinin Titer, Mouse-Protective Value, and Rabbit Therapeutic Value of Seven Sera.

Serum	Mouse-protective value (in terms of fatal doses protected against by 1 cc. of serum, if serum and organisms are mixed *in vitro* before injection)	Rabbit therapeutic value		Agglutinin titer
		Minimal effective dose of serum (amount necessary to bring about recovery if administered 24 hrs. after infection)	Minimal effective doses per cc. of serum	
		cc.		
1	500,000,000	3.0	0.33	80
2	50,000,000	3.5	0.29	40
3	50,000,000	3.5	0.29	40
4	500,000	>10.0	Less than 0.1	0
5	1,428,000,000	0.2	5.0	640
6	5,000,000	3.5	0.29	10
7	50,000,000	0.5	2.0	320

the condition in man, under which serum is therapeutically employed, as closely as this can be done in animal experiments.

It is not altogether surprising that there is not a complete parallelism between figures in the three columns but it is somewhat difficult to account for this in every case. The first point that may well be considered is the therapeutic value of homologous as against heterologous immune sera. Nos. 6 and 7 are from rabbits, and it is to be observed that these sera have a low mouse-protective value in comparison with their relatively high therapeutic value for rabbits. Thus, Serum 7 has only one-tenth the protective value of Serum 1, but its

therapeutic value is six times greater. Compared to Nos. 2 and 3, which have the same protective value, this serum has six or seven times the therapeutic value. The same comparisons hold good for Serum 6. It is obvious that an homologous immune serum, although low in protective value for an heterologous animal, does possess marked therapeutic value as contrasted to the immune serum of a third species. The cells of the treated animal are intimately concerned with any change in the status of the disease and the homologous serum probably owes its advantageous effect to the fact that such proteins are more readily absorbed by the cells.

A consideration of the therapeutic value of the four more potent antisera from horses (Nos. 1, 2, 3, and 5) shows that there is a more definite correlation with the agglutinin titer than with the mouse-protective value. There is a very striking dissimilarity in relative mouse-protective and rabbit therapeutic values.

It is suggested that a method of standardization of antipneumococcic serum, based on the rabbit therapeutic usage as outlined, might prove valuable as compared with the present system. Such a procedure would be no more difficult and only slightly more expensive than the mouse method and the results are quite as regular and definite. It would have the great advantage of measuring that property for which the serum is utilized. Our chief hesitancy in proposing such a method is that we do not know, nor is there any present method of determining, whether the therapeutic value for rabbits does represent a definite measure of therapeutic value in human lobar pneumonia.

II.

Non-Specific Therapy.

In many of these therapeutic experiments large amounts of foreign protein have been injected and it becomes important to separate the specific and non-specific effects. To this end the latter type of therapy has been studied in sixteen cases with normal horse and rabbit sera and typhoid vaccine as the non-specific agents. Various quantities were employed and the cases were usually treated at 24 hours following infection. Four of this series of sixteen survived but this survival rate does not sufficiently exceed that of untreated cases to be considered significant.

Following the intravenous injection, at 24 hours after infection, of 3 to 5 cc. of normal horse serum the temperature may be slightly elevated for a few hours. The number of white cells, already rather low, is not appreciably altered. The most striking result that has been
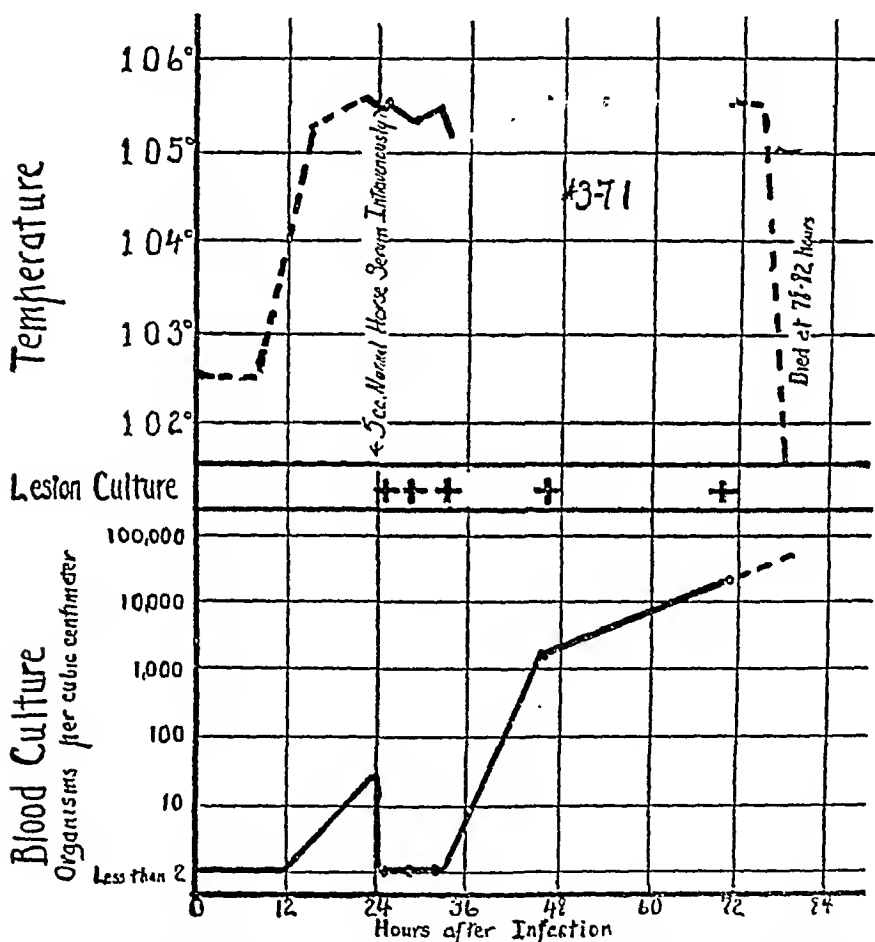
FIG. 2. Chart of findings on Rabbit 3–71 showing the effects of the injection of a non-specific agent.

observed is the immediate disappearance of pneumococci from the circulating blood. The blood may remain free of organisms for several hours but the bacteria then reappear in increasing numbers. A chart of such a case is shown in Fig. 2.

The normal horse serum used in these experiments had no protective action against pneumococci in mice and the result of the treatment of these rabbits, in so far as can be determined, must be due entirely to non-specific factors. This conclusion is supported by the finding that the same result is obtained if typhoid vaccine is administered intravenously.

It has been suggested that the immediate freeing of the blood stream of organisms in the specifically treated case may be due entirely to non-specific factors, but this is probably not the case. That the specific element alone is capable of bringing about this effect is shown in instances of successful intravenous therapy with the homologous (rabbit) immune serum as contrasted to entirely negative results with the homologous normal serum. The injection of normal rabbit serum appears to have no effect upon the number of circulating organisms.

These results show that the non-specific element involved in specific intravenous therapy with "raw" serum must be considered as a definite factor but negligible as compared to the specific element and probably having no bearing on the outcome of any case.

III.

Active Immunity.

In our previous communication (1) the relation of crisis to the development of active immunity in the experimental rabbit disease was discussed. In the following paragraphs we propose to present observations concerning active immunity in convalescent animals and in those previously vaccinated, and to discuss the problem of active immunization in pneumonia.

The Determination of Active Immunity.—Our experience leads us to believe that for the present the only reliable method of determining active immunity is by observing the response to reinfection. Preliminary experiments showed that convalescent rabbits, though immune, still develop a limited localized lesion at the site of intradermal inoculation of undiluted virulent pneumococcus broth culture, and for this reason, before an arbitrary reinfection dose could be selected, it was necessary to study the results obtained with varying amounts of culture.

The method of carrying out this experiment differed from that used in determining the susceptibility of the normal rabbit to varying intradermal dosage of pneumococci, for in the convalescent case the lesion obtained does not exhibit the tendency to spread that is seen in the normal animal.   A number of tests may therefore be made on a single animal at the same time.   The results of such an experiment are shown in Table II.

On the basis of this experiment it seemed that there would be no particular advantage attached to the use of any one dose for reinfec-

TABLE II.

Susceptibility of the "Immune" Rabbit to Varying Dosage of Pneumococci.

Rabbit 4–28; 22 days convalescent.   Entire abdominal area shaved, and varying amounts of culture, diluted to 0.2 cc. in broth, injected intradermally in various separated areas.

Area	Amount of broth culture	Reading at 24 hrs.
	cc.	
1	0.2	Local lesion with definite color and edema; no tendency to spread
2	0.04	As above
3	0.01	Local area of heightened color; no swelling
4	0.002	Trace of color at point of inoculation; no swelling
5	0.001	Very slightest trace of color at point of inoculation; no swelling

With amounts below those given, no sign of inflammation was observed

tion except that with the larger amounts the local lesion would amply safeguard the fact that virulent organisms had been injected.   We have therefore used 0.2 cc. of undiluted 18-hour broth culture as a routine.   This is injected, as in cases described in our previous paper, well up on the animal's side.   Observations as to the character of the lesion and the animal's temperature are made at 24 hours.   Blood cultures are not done unless the lesion is widespread, for in our experience the local type of lesion is associated with entirely negative blood cultures.

The resistance to infection has been studied by this method in over 50 cases.   The results show some variation but each case falls dis-

mental disease, it has not been possible to study some features of the acquired immunity in the greatest detail. It has been determined that a definite $(+++)$ immunity is acquired and that this immunity persists at a high level for several months. In one case it persisted for 9 months but in two other instances the immunity was not maintained for this length of time. In one case in a series of ten, partial immunity only was observed at the end of the 2nd month. We hope to determine the limits of efficiency of this acquired immunity in future experiments. For the present no absolute conclusion is justified except that the immunity acquired seems to persist for several months but is not permanent.

Active Immunity in Treated Cases.—In repeated instances we have determined that if a rabbit subjected to dermal inoculation is treated within 24 hours with an effective dose of heterologous serum it promptly recovers but after the passive immunity has worn off, such an animal possesses no active immunity against subsequent infection. We have reinfected rabbits of this type at from 15 to 60 days following original infection and in no case have we observed more than a "one-plus" immunity. In several cases the immunity was entirely negative.

If treatment is delayed until the 48th or 50th hour it does not appear to interfere with the development of active immunity, for such cases show the characteristic "three-plus" immunity after recovery.

This same principle has been demonstrated in animals given a single vaccination of washed heat-killed pneumococci. No active immunity develops in such cases if they are given, within 24 hours after vaccination, an amount of heterologous immune serum corresponding to an effective dose for that hour.

It has been suggested that this arrest of developing immunity may be concerned with the lack of immunizing power of the substances resulting from the proteolytic digestion of the phagocyted bacteria. It seems certain that the phagocytic activity of circulating leucocytes is increased by the agency of the immune serum and in the case of early treatment it might be reasoned that the bacteria or their antigenic fractions do not reach the tissues in form suitable for stimulating antibody production.

If cases are treated with heterologous immune serum in amounts insufficient to produce therapeutic effect (subeffective) a certain

number of animals recover spontaneously after a typical and severe course. In these instances the introduction of subeffective amounts of serum had not interfered with the development of immunity and these cases show the same immune character as do the untreated cases.

If an homologous antipneumococcic serum is used for arresting the course of the disease, and the rabbits are subsequently tested for immunity, the results differ from those obtained when heterologous serum is used. These animals, treated with the homologous serum, show a definite $(+ + +)$ immunity following recovery. This immunity persists certainly for over a month at a high level and in one case a slight immunity $(+)$ remained at 9 months. At this time there is no method by which it can be determined whether this result is due to an active immunity or to a prolonged and effective passive immunity. In this connection it is of interest to recall that Park (5) has reported a similar finding as regards diphtheria antitoxin, for he found that a guinea pig receiving 10 units of diphtheria antitoxin prepared in an immunized guinea pig remained immune for from 6 to 8 months, while a guinea pig receiving 10 units of antitoxin made in an immunized horse, remained immune for only 2 or 3 weeks.

Active Immunity and Agglutinins.—In twenty-eight cases of complete immunity to reinfection we have observed circulating agglutinins in about half. Partially immune animals have also been observed to show circulating agglutinins, even in as high a titer as 1–40. It is not easy to appraise the significance of these results, which however indicate quite clearly that the absence of circulating agglutinins does not signify the lack of immunity, and conversely it is also probable that the presence of circulating agglutinins does not indicate that the animal is completely resistant to infection.

Active Immunity and the Mouse-Protective Value of the Serum.—In a series of some twenty-five recovered rabbits we have correlated the immunity against infection with the protective value for mice of serum taken at that time. Animals whose sera are protective have always shown some degree of immunity to infection and in most instances this immunity is complete. Animals which are entirely non-resistant to infection show no protective substance but it is also true that some rabbits which are completely immune may possess serum with no protective value.

From the protective property alone, no conclusion is justified as to the immunity possessed by the animal. There remains only one method of testing the immunity of the experimental animal and that is to reinfect it.

Active Immunity after Vaccination with Killed Pneumococci.—It has been stated above that the "hyperimmunized" rabbits usually show a complete immunity (++++) against infection if such a test is carried out during the time that the antibody titer is high.

In our preceding paper we had occasion to use single vaccination as a means of studying the possible significance of the crisis. The knowledge that a single vaccination gave rise within 5 days to an immunity sufficient to protect an animal against infection has continued to interest us in its possible application to the disease in man. For this reason we have begun to study the immunity acquired in rabbits as a result of a single vaccination with heat-killed pneumococci. For routine intravenous vaccination we have used 10 cc. of a suspension, of washed heat-killed pneumococci, the density of which was 1.2 cm. (as measured by the method of Gates (6)). This is an extremely heavy suspension.

If the vaccinated rabbits are tested at 5 days by the usual method of intradermal inoculation of 0.2 cc. of undiluted broth culture, a three- or four-plus immunity is observed, in that respect being quite analogous to the convalescent case. In another sense the one-vaccination animal is unlike the convalescent for its acquired immunity wears off somewhat more quickly. In only one instance, of eight cases, did it persist in maximum degree for as long as 2 months.

The development of protective substances (for mice) in the rabbit has been quite thoroughly studied by Armstrong (7). In many of his experiments he observed a rise of protective substances within 3 to 5 days after a single injection of pneumococcus vaccine. We have confirmed that result. In Armstrong's series there was a gradual drop of circulating protective substances in from 8 days to several weeks; these intervals he held to be somewhat proportional to the amount of vaccine used. In our experiments this drop of protective substances has been irregular and apparently independent of the dosage.

The development of protective substances and immunity during a

period of 5 or 6 days following vaccination seems to be a characteristic of the pneumococcus rather than of the species of the animal used. We have found that mice develop an active immunity against infection within 4 or 5 days after a single injection of vaccine and this is possibly the fundamental reason that makes it unnecessary to observe mice in protection experiments for longer than that period of time. Through the kindness of Drs. White and Robinson of the Massachusetts Antitoxin and Vaccine Laboratory it has been possible to study the development of protective substances in horses after a single injection of heat-killed pneumococci. The sera of these horses were not examined on the 5th day but on the 6th protective substances were found in each of two cases. The crisis in human lobar pneumonia at 5 to 7 days must also be correlated with this characteristic period of time.

The Possible Application of These Findings.—In the foregoing paragraphs it has been established that, in rabbits, active immunity, whether acquired in disease or artificially by vaccination, is not of exceedingly long duration, and even at its height is unable in all cases effectively to overcome overwhelming doses of organisms immediately (the typical three-plus immunity). These facts are comparable to the present theories regarding active immunity in human lobar pneumonia.

There have been attempts, especially by Lister (8), and by Cecil and Austin (9), to use vaccination as a means of prophylaxis. Considering the difficulties in the preparation of a polyvalent vaccine and the apparently low antigenic properties of Type III, the results reported by these workers were very encouraging.

Although our experiments have been entirely with Type I and with experimental animals, it would seem that if vaccination could be applied in a systematic way to a susceptible population throughout the "pneumonia season" it should yield results. In such an application the two factors that must be especially considered are (a) the polyvalent character of the vaccine and (b) the shortness of the immune phase conferred by vaccination. It would seem possible to rule out the second point by the use of single subcutaneous injections of vaccine at 1- or 2-month intervals during the season of highest pneumonia morbidity.

SUMMARY.

1. The continuation of our experiments with intradermal Type I pneumococcus infection in rabbits has furnished further evidence of the marked analogies between this condition and that of human lobar pneumonia.

2. It has been found that the amount of antiserum necessary for successful therapy increases as the disease progresses, and that this progression has a definite mathematical character. Such a condition, it seems, can only be caused by a progressive accumulation of some toxic or antagonistic substance, the exact nature of which is not known.

3. Various lots of antipneumococcus sera have been tested for their therapeutic properties. The results from seven such sera show that this therapeutic value does not parallel the mouse-protective value. It is suggested that the rabbit technic may prove useful for the routine comparison and standardization of antipneumococcus sera since it represents a simple method for determining that property for which the serum is to be utilized.

4. The effect of non-specific therapy in this condition has been determined to be a transient disappearance from the blood stream of circulating organisms. This result was obtained with such heterologous materials as normal horse serum and typhoid vaccine but not with the homologous normal rabbit serum.

5. Rabbits recovering from the intradermal disease without treatment or with such inadequate treatment that the disease runs its normal course, were shown to have a definite though not permanent immunity. Cases in which the disease had been arrested at 24 hours by effective therapy with heterologous immune serum showed no immunity after the early disappearance of the passively administered elements. Cases which were brought to early recovery with immune homologous serum did show a definite immunity comparable to that which was developed in other animals as the result of an untreated course of the disease.

6. The immunity conferred by single and multiple vaccination is reported. The possibility of the application of such methods in the pneumonias of man is discussed and a method for such an application is suggested.

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THE RÔLE OF STREPTOCOCCI IN EXPERIMENTAL POLIO-MYELITIS OF THE MONKEY.

By PERRIN H. LONG, M.D., PETER K. OLITSKY, M.D., AND FRED W. STEWART, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Within the last 12 years, several investigators have reported the isolation of streptococci from poliomyelitic tissues of man and of animals. These workers, in view of the ease with which the streptococci could be recovered and the supposed fulfillment by the organisms of one or all of Koch's postulates, believe that the streptococci are either the incitants of, or are etiologically related to poliomyelitis.[1–4] It was stated, furthermore, that the microorganisms after repeated injection into horses, can produce a serum which has neutralizing, protective, and therapeutic properties in poliomyelitis.[5] Other experimenters, however, have failed to confirm this opinion.

Thus Bull[6] reported that streptococci found in cultures of brain and spinal cord from poliomyelitic monkeys induced in monkeys neither the clinical signs

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[5] Rosenow, E. C., *J. Am. Med. Assn.*, 1917, lxix, 261, 1074; *J. Infect. Dis.*, 1918, xxii, 379. Nuzum, J. W., *J. Am. Med. Assn.*, 1917, lxviii, 24. Nuzum, J. W., and Willy, R. G., *J. Am. Med. Assn.*, 1917, lxix, 1247; *J. Infect. Dis.* 1918, xxii, 258.

[6] Bull, C. G., *J. Exp. Med.*, 1917, xxv, 557.

nor the histopathological lesions of the experimental disease which follows inoculation of the filtered poliomyelitic virus. Nor were monkeys protected against filtered virus after recovery from the effects of injection of streptococci. Bull also cultivated streptococci from the tonsils of man—a tissue frequently employed by others[1,2,4] as a source of the supposed cocci of poliomyelitis. Streptococci from the tonsils of 32 patients with poliomyelitis and similar bacteria from the tonsils of non-poliomyelitic patients showed no differences in their respective action in laboratory animals. Bull, therefore, regarded the streptococci as secondary bacterial invaders of nervous tissue.

Smillie[7] concluded that the streptococcus could not be implicated in the pathology of the poliomyelitic process. He found that it was a common contaminant, or a secondary invader: streptococci occurred in animals which were etherized while moribund, or which had died some hours before autopsy, but were absent in virus-infected animals which were killed while still relatively strong. Amoss[8] maintained a similar attitude.

With reference to the specific neutralization of the poliomyelitic virus by streptococcus immune serum, reported by Rosenow,[5] by Nuzum and coworkers,[5] Amoss and Eberson[9] found that such serum had neither neutralizing powers *in vitro* nor *in vivo*, or any therapeutic property in monkeys.

Finally, Flexner and his coworkers,[10] Levaditi and Landsteiner,[11] and others[12] have found that the effects of the filtrable poliomyelitic virus could not be ascribed to the action of ordinary bacteria.

The discrepancy in the results of different investigators and the recent increased interest in the use of antistreptococcic serum for the prevention and treatment of infantile paralysis, led us to a restudy of cultivation of poliomyelitic tissues. The study concerned especially the source of streptococci and their relation to the etiology of the disease. In general, it included a comparison of strains of streptococci isolated from monkeys affected with experimental poliomyelitis;

[7] Smillie, W. G., *J. Exp. Med.*, 1918, xxvii, 319.

[8] Amoss, H. L., in Rivers, T. M., Filterable viruses, The Williams and Wilkins Company, Baltimore, 1928, 173.

[9] Amoss, H. L., and Eberson, F., *J. Exp. Med.*, 1918, xxvii, 309; 1918, xxviii, 323.

[10] Flexner, S., and Lewis, P. A., *J. Am. Med. Assn.*, 1909, liii, 2095; see also Bull.[6]

[11] Levaditi, C., and Landsteiner, K., *Compt. rend. Soc. biol.*, 1910, lxviii, 311.

[12] Zappert, J., v. Wiesner, R. R., and Leiner, K., Studien über die Heine-Medische Krankheit (Poliomyelitis Acuta), Leipsic and Vienna, 1911, 137. Kling, C., Petterson, A., and Wernstedt, W., *Communications Inst. méd. Etat Stockholm*, 1912, iii, 5.

with 3 strains of microorganisms generously supplied by Dr. Rosenow, and derived from poliomyelitic tissues; with 1 strain obtained from the American Type Culture Collection, also derived from poliomyelitis; and with 3 cultures of Rosenow poliomyelitic streptococci supplied by Eli Lilly and Company, whose kindness is gratefully acknowledged.   In addition, cultures were made from the central nervous systems of poliomyelitic monkeys, with certain media recommended by Rosenow and others, media in which they obtained the so called poliomyelitic streptococcus.   In view of the findings of two of the writers (Long and Olitsky[13]) that in so far as herpes virus encephalitis of rabbits and guinea pigs is concerned, the process of grinding permits contamination of tissues with streptococci and other ordinary bacteria, we cultured both fragments and emulsions of ground material from the same brain. Furthermore, cultivation tests were made with portions of the same brain in different rooms so as to check the results of one series against those of the other.

Mode of Procedure.

The materials cultured, the media and methods employed were as follows:

Source of Virus.—The poliomyelitic virus used consisted of the brains of 17 monkeys which showed the typical signs and pathological lesions of experimental poliomyelitis induced by the injection of either the M.A. strain[14] of poliomyelitic virus, or virus supplied us by Dr. Aycock.[15]   The animals were killed at a time when the characteristic flaccid paralyses were clearly evident,[16] and tissues were removed for culture.   In a few instances, the tissues were procured after the animal died from the typical experimental disease.   In all cases, the diagnosis was checked by finding characteristic histopathological changes in the brain and spinal cord.[17]   In addition the brain of a monkey which died from a non-poliomyelitic affection was also cultured.

The brains were removed from the monkeys under sterile precautions, and when fragments were used for cultures, 5 mm. cubes of tissue were cut for the purpose.   When emulsions were employed, a portion of the brain was ground by

[13] Olitsky, P. K., and Long, P. H., *J. Exp. Med.*, 1928, xlviii, 199.

[14] Flexner, S., Clark, P. F., and Amoss, H. L., *J. Exp. Med.*, 1914, xix, 195.

[15] This strain was more active in monkeys than the M.A. strain.

[16] Full ether anesthesia was used in all experiments.

[17] Flexner, S., and Lewis, P. A., *J. Exp. Med.*, 1910, xii, 227.

means of a sterile mortar and pestle. To this, physiological saline solution was added to make a 10 per cent suspension. About 1 cc. of the suspension comprised the inoculum. It is to be emphasized that all procedures were carried out under the strict conditions underlying sterile bacteriological technique.

Media.—The media employed consisted of:

(1) 5 per cent rabbit's blood, beef infusion 1 per cent dextrose or plain agar in Petri dishes.

(2) Chopped meat medium, prepared according to the directions given by Evans.[18] The medium was used because it was employed by the latter to recover streptococci from epidemic encephalitis in man and experimental encephalitis in monkeys and rabbits.

(3) Ascitic fluid-dextrose broth medium in long tubes. The medium was prepared according to the directions of Rosenow and Towne.[19]

(4) Smith-Noguchi medium based on the principles outlined by Gates and Olitsky.[20]

(5) Hartley's modification of Douglas' tryptic digest broth.[21] The special broth served as a basis for the study of toxins of the streptococci isolated during the course of the experiments.

(6) Beef infusion broth either plain or containing 1 per cent dextrose, for the preparation of agglutinogens and for a study of cultural reactions.

(7) Sterile peptone solution containing the Andrade indicator and respectively 1 per cent of dextrose, maltose, lactose, saccharose, raffinose, salicin, inulin, and mannitol for fermentation reactions.

Plan of Experiments and Technique.—For the purpose of ascertaining the origin of microorganisms in the cultures, that is, whether they were derived from the tissues themselves or from sources outside the tissues, experiments were designed as follows: (*a*) the same material was cultured in different media all of which were favorable for the growth of streptococci; (*b*) one brain was subdivided under sterile precautions into two parts of which one portion was used for cultures of fragments and the other for cultures of emulsions; (*c*) to eliminate the personal factor in the results obtained, two of the writers separately cultured portions of the same material; (*d*) one series of tests was made under the usual conditions of sterility, that is, under a hood in the room used for routine work. Another series was made under extraordinary conditions of sterility: cultures were performed in a manner similar to that employed by Dr. Carrel at The Rockefeller Institute for inoculation and transplantation of living tissue cells. The worker clothed himself in sterilized gown, hood, face mask, and rubber gloves. The media,

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[18] Evans, A. C., *Pub. Health Rep., U. S. P. H.*, 1927, xlii, 171.

[19] Rosenow, E. C., and Towne, E. B., *J. Med. Research*, 1917, xxxvi (N.S. xxxi), 175.

[20] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[21] Hartley, P., *J. Path. and Bact.*, 1922, xxv, 479.

material for cultivation, and instruments for cutting and grinding tissues were kept in sterile towels and treated as is done in modern surgical aseptic procedures. The room was especially adapted for elimination of air contaminants. It had no connection with the outside air and prior to the entry of the worker, motor-driven, powerful water sprays were put into action for the purpose of clearing the atmosphere of particles. The effectiveness of the procedures is exemplified by the fact that on one occasion blood agar medium in Petri dishes kept open for 35 minutes revealed no colonies of microorganisms.

All cultures were incubated at 37°C. and whenever any tube showed evidence of growth, it was examined; negative tubes were retained for 14 days before a final reading was made. It may be stated here that growth of the bacteria occurred as a rule from 18 to 72 hours after inoculation.

Cultivation Experiments.

Emulsions.—The first series of experiments comprised cultures of emulsions of brains from 18 monkeys in chopped meat medium and in ascitic dextrose broth. These were made by two of the writers, one working under the hood in the room usually employed for routine work, and the other in the special room already described, one worker alternating with the other in different tests. In two of the experiments a third person substituted for one of the original group.

The results of this experiment indicate that when the cerebral tissue was ground and emulsified and this material used for culture, a number of microorganisms, of different species, could be recovered. The number but not the species could be influenced by environmental conditions: when the usual, although strict sterile precautions were followed, the percentage of tubes of ascitic dextrose broth medium positive was 60, and of chopped meat medium, 57. When extraordinary care for sterility was employed, the positive tubes were respectively 21 and 13 per cent of all in each series.

Further analyses of Tables I and II show that the bacteria commonly encountered in the cultures of poliomyelitic brain emulsions in ascitic broth and meat media were diphtheroids, staphylococci, non-hemolytic streptococci, and spore-bearing rods. The same species were found on the medium in the Petri dishes exposed to the atmospheres of the two environments in which the cultures were made. The average time of exposure was about 30 minutes, that is, during the course of the setting up of a particular series of cultures. The average number of colonies on a plate exposed under the hood was

TABLE I.

Emulsions of Brain Cultured in Ascitic Dextrose Broth.

Monkey No.	Status	No. tubes		No. negative		No. positive		Non-hemolytic streptococci		Diphtheroids		Staphylococci		Spore-bearing rods		Miscellaneous		No. of colonies on opened plates	
		H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
1	Experimental poliomyelitis	6		0		6		4		0		3 (a)		0		0		I(c) = 25; II = 17	
2	"	9	6	5	6	4	0	0	0	2	0	2	0	0	0	0	0	I = 59; II = 47	I = 0
3	"	6	6	1	3	5	3	0	0	4	0	0	3	0	0	1 (b)	0	I = 52; II = 42	I = 6; II = 10
4	"	6	6	4	6	2	0	0	0	1	0	1	0	0	0	0	0	I = 16; II = 16	I = 2; II = 2
5	"	5	6	0	5	5	1	0	0	4	1	1	0	0	0	0	0	I = 18; II = 16	I = 2; II = 4
6	"	5	5	3	4	2	1	1	0	2	1	0	0	0	0	0	0	I = 14; II = 8	I = 2; II = 6
7	"	6	6	4	6	2	0	1	0	0	0	1	0	0	0	0	0	I = 16; II = 11	I = 5; II = 0
8	Normal	6	6	2	0	4	6	0	6	1	0	0	0	1	0	1 (d)	0	I = not counted; II = 12	I = 4; II = 2
9	Experimental poliomyelitis	5		3		2		0		0		2		0		0			
10	"	6		4		2		0		1		1		0		0			
11	"	6		2		4		0		3		1		0		0			

																	I =/II =	I =/II =
12	"	6	1	6	5	0	0	5	0	1	0	0	0	0	0	I = 12 / II = 16	I = 1 / II = 12
13	"	6	0	5	6	1	0	5	0	0	1	0	0	1 (e)	0	I = 35 / II = 37	I = 0 / II = 0
14	"	6	2	4	4	2	0	2	2	1	0	1	0	0	0	I = 17 / II = 15	I = 3 / II = 0
15	"	6	5		1	1	0	0	0	0	0	0	0	1 (f)	0	I = 12 / I = 8	I = 0 / II = 1
16	"	6	5	5	1		0	0	1	1	0	0	0	0	0	II = 10	
17	"	6	0	6	6	0	0	3	0	3	0	0	0	0	0	I = 12 / II = 12	
18	"	6	2		4	0	1	3 (g)	0	0	0	0	0	1 (h)	0	I = 14 / II = 7	
Total 18		108 71	43	56	65	15	7	6	36	5	18	4	2	0	5	0	20+	3 —
Per cent =			39+	78+	60+	21+											Average No. of colonies =	

(a) In one case admixed with streptococci. (b) M. tetragenus. (c) No. of plate indicated by Roman numeral. The variations in numbers of colonies is due to length of time of exposure to the air which lasted throughout the period of the test. (d) Streptobacillus. (e) Diplobacillus. (f) Hemolytic streptococcus. (g) In one case admixed with streptococci. (h) Mould. II = cultures made in room used for routine work; C = cultures made in special (Dr. Carrel's) room.

about 20 and in Dr. Carrel's tissue culture room, about 3. There was evidently a correlation between the number of positive cultures with the number of bacteria in the air.

TABLE II.

Emulsions of Brain Cultured in Chopped Meat Medium.

Monkey No. (a)	No. tubes		No. negative tubes		No. positive tubes		Non-hemolytic streptococci		Diphtheroids		Staphylococci		Spore-bearing rods		Miscellaneous	
	H	C	H	C	H	C	H	C	H	C	H	C	H	C	H	C
1	6		0		6		4		1		1		0		0	
2	9		3		6		1		2 (b)		4 (c)		1 (b)		0	
3	6	6	1	6	5	0	1	0	3	0	0	0	0	0	1 (d)	0
4	6	6	5	4	1	2	0	0	0	0	1	2	0	0	0	0
5	5	7	1	7	4	0	0	0	4	0	0	0	0	0	0	0
6	5	6	0	6	5	0	0	0	1	0	3	0	1	0	0	0
7	6	5	2	4	4	1	1	0	1	1	1	0	1	0	0	0
9	5	6	4	5	1	1	0	0	0	0	1	0	0	1	0	0
10	6	6	4	2	2	4	0	4	0	0	2	0	0	0	0	0
11	6		5		1		0		1		0		0		0	
12	6	6	1	6	5	0	0	0	0	0	5	0	0	0	0	0
13	6	6	4	6	2	0	0	0	2	0	0	0	0	0	0	0
14	6	6	6	6	0	0	0	0	0	0	0	0	0	0	0	0
15	6		1		5		0		1		3		0		1 (e)	
16	6	6	4	5	2	1	0	0	2	1	0	0	0	0	0	0
17	6		0		6		0		2		4		0		0	
Total 16	96	66	41	57	55	9	7	4	20	2	25	2	3	1	2	0
Per cent =			42+	86+	57+	13+										

(a) For status of monkeys see Table I. Since these tests were made at the same time as those recorded in Table I, the control air cultures are not repeated here. (b) In one instance admixed with spore-bearing rod. (c) In one instance admixed with streptococci. (d) *M. tetragenus.* (e) Streptothrix. H and C as in Table I.

In addition it will be noted that although two portions of the same poliomyelitic or non-poliomyelitic monkey brain were employed, the results of cultures of each portion were in most instances wholly dissimilar. A striking example is that of Monkey 10. In one environment, 10 of 12 tubes of medium yielded non-hemolytic streptococci

(C, Tables I and II); in another room, of 12 tubes, 1 tube showed diphtheroids, 3 tubes staphylococci, and none revealed streptococci (H, Tables I and II).

Since the brains for culture were removed from monkeys while most of them were still relatively strong, although in different stages of flaccid paralyses, the notion that the bacteria were agonal invaders was not tenable. Moreover, the ground brain of the non-poliomyelitic monkey which was cultured at autopsy in ascitic broth also exhibited in different tubes diphtheroids, spore-bearing rods, diplobacilli, and non-hemolytic streptococci.

TABLE III.

Cultures of Fragments of Monkey Brain in Ascitic Broth and Chopped Meat Medium.

Monkey No.	Status	No. tubes chopped meat medium		No. tubes ascitic dextrose broth		No. of tubes positive in the meat medium		No. of tubes positive in the broth	
		H	C	H	C	H	C	H	C
2	Experimental poliomyelitis	9				0			
7	" "	6	5	5	6	0	0	0	0
8	Normal	6		6		0		0	
9	Experimental poliomyelitis	5	6	5	6	0	0	0	0
16	" "	6			6	0			0
17	" "	6			6	0			0
18	" "			6				1 (a)	
Total 7		38	11	22	24	0	0	1	0
	Total No. tubes 95				Total positive 1 tube				

(a) Diphtheroids. H and C as in Table I.

In general, the results of cultivation of emulsions of ground poliomyelitic and non-poliomyelitic cerebral tissues of monkeys parallel closely those of cultivation from herpes virus encephalitis of rabbits and guinea pigs, already reported.[13] In the last mentioned disease, it was shown that the various bacteria of common species, including the streptococci, were contaminants introduced during the process of grinding. Furthermore, in herpes virus encephalitis no growth of microorganisms was obtained when fragments, instead of ground

material, were used.   How this applies in turn to poliomyelitic brain cultures is illustrated by Table III.

Fragments.—7 of the monkey brains which were cultured in the form of ground material were also cultured in the form of fragments.

Table III shows that of a total number of 95 tubes containing ascitic dextrose broth or chopped meat medium and fragments of monkey brains, 6 poliomyelitic and 1 non-poliomyelitic, only 1 exhibited growth.   The positive tube contained diphtheroids. If a comparison be made with the cultures of emulsions of the same brains and the same media (Tables I and II), it will be plain that with poliomyelitic brains just as with herpes virus encephalitis brains, cultures of ordinary bacteria occurred as a consequence of emulsifying the cerebral tissues.   Again it should be emphasized that the results shown in all three tables were derived from portions of the same brain.

In an additional experiment, a poliomyelitic brain from Monkey 15 was cut into fragments, the fragments placed in broth at 37°C. for 24 hours, and then transferred to 6 tubes of ascitic dextrose broth and 6 tubes of chopped meat medium.   The latter medium was, after 14 days incubation, negative but the ascitic broth cultures showed non-hemolytic streptococci in 2 tubes, staphylococcus, diphtheroids, and streptothrix in single respective tubes.   This test was made in the routine culture room.   It should be compared with the findings in the same material as given in Tables I and II.   It is obvious that the results in this case depended on the additional handling of the brain.

To summarize, a variety of ordinary bacteria including non-hemolytic streptococci can be recovered from cultures of poliomyelitic monkey brains.   They appear irregularly in tests with different portions of the same brain, occurring sometimes in one favorable medium but not in another, frequently in one room but not in a second, often when only the usual bacteriological technique for sterility is followed but much less often when extraordinary precautions for sterility are maintained, and generally in emulsions of the brain but not in its fragments.   Furthermore, the bacteria are similar to those often recovered from the air of the place in which the cultures are made. What has been said, therefore, of herpes virus encephalitis can be stated again as applying to experimental poliomyelitis in monkeys, namely, the bacteria are introduced into the material for culture during grinding.[13]

Smith-Noguchi Medium.—This opinion is further supported by results of cultures in the Smith-Noguchi medium, although the primary purpose of this series of cultivation tests was the recovery of the globoid bodies of Flexner and Noguchi.[22] The cultures were made with fragments of monkey brain in ordinary Smith-Noguchi medium[20]

TABLE IV.

Cultivation of Fragments in Smith-Noguchi Medium.

Monkey No. (a)	No. of tubes		No. of tubes positive		Diphtheroids		Non-hemolytic streptococci		Staphylococci		Miscellaneous		Globoid bodies	
	O	B	O	B	O	B	O	B	O	B	O	B	O	B
3	5	5	1	0	1	0	0	0	0	0	0	0	0	0
4	6	6	1	6	0	2	0	0	1	0	0	1 (b)	0	3
5	5	5	4	0	1	0	1	0	1	0	1 (c)	0	0	0
6	5	5	2	1	1	0	0	0	1	0	0	1 (d)	0	0
7	6	6	1	2	1	1	0	0	0	0	0	1 (c)	0	0
8	6	6	4	1	2	0	0	1	0	0	2 (c)	0	0	0
9	6	6	0	0	0	0	0	0	0	0	0	0	0	0
10	6	6	0	0	0	0	0	0	0	0	0	0	0	0
11	6	6	0	1	0	1	0	0	0	0	0	0	0	0
12	6	6	0	0	0	0	0	0	0	0	0	0	0	0
13	4	5	1	1	0	0	0	0	0	0	0	0	1	1
14	6	6	0	0	0	0	0	0	0	0	0	0	0	0
16	6	6	0	0	0	0	0	0	0	0	0	0	0	0
17	6	6	0	1	0	1	0	0	0	0	0	0	0	0
Total 14	79	80	14	13	6	5	1	1	3	0	3	3	1	4

(a) The status of these monkeys is given in Table I. Compare with Tables I to III for results on aerobic cultivation. (b) Streptothrix. (c) Spore-bearing rod. (d) Diplococcus. O = Ordinary Smith-Noguchi medium and B = Boëz' apparatus.

and similar medium having no petrolatum seal but placed in an anaerobic apparatus described by Boëz.[23] The results are given in Table IV.

There is general agreement on the difficulty of avoiding contamination in the Smith-Noguchi technique. In the series shown in Table IV 27 of 159 tubes exhibited different bacteria. The number of positives

[22] Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

[23] Boëz, L., *J. Bact.*, 1927, xiii, 227.

might have differed if one instead of three workers had made all the tests. However, in the case of 2 monkeys (Nos. 5 and 6), emulsions of ground brain were also planted in Smith-Noguchi medium; 10 of 12 tubes showed different bacteria.

With respect to the isolation of globoid bodies, it is to be noted that they were obtained from the poliomyelitis brains of 2 of the 14 monkeys (Table IV). In 1 case, 3 of 12 tubes were positive, and in the other 2 of 9 tubes. 1 culture in its first generation was inoculated intracerebrally into a monkey; no signs of experimental poliomyelitis developed.[24]

The globoid bodies were found to be distinct microorganisms but differed from the streptococci recovered in the tests and from those obtained from Rosenow. They were definitely anaerobic; indeed, in one instance, a transplant from a culture in ascitic agar kept in the dark at room temperature for about 9 years was still viable and anaerobic. On the other hand, the inoculation of streptococci into Smith-Noguchi medium failed to bring about their conversion to globoid type. 9 strains of streptococci, including 3 of Rosenow's series, planted in the medium and examined after 14 days incubation showed death of 2, somewhat smaller forms in 3, and no change in the remaining strains. Transfer of the survivors to aerobic medium yielded profuse growths of typical large cocci or coccoid forms. These conditions prevailed through 3 successive subplants in Smith-Noguchi medium at 14 day intervals.

Properties of the Streptococci.

31 strains of non-hemolytic streptococci were collected for comparative study. In this collection were 7 strains of Rosenow, all of which were stated to be derived from poliomyelitis;[25] 1 was recovered from "ground" broth and 3 from ground chopped meat

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[24] In this connection it is of interest to record a repeated experiment in which fragments of poliomyelitic brain were placed in broth and kept for 14 days under aerobic and anaerobic conditions. At the end of this time, the fragments were removed and ground in saline solution and injected intracerebrally in monkeys. Neither the aerobic nor the anaerobic material was found to be active.

[25] 3 of the strains were given us by Dr. Rosenow, 3 by Eli Lilly and Company, and 1 was obtained from the American Type Culture Collection.

medium, already described;[13] 2 from herpes virus encephalitis in rabbits;[13] 2 from normal guinea pig brain;[13] 5 from the air in the rooms in which the cultures were made; 1 from a non-poliomyelitic monkey brain; and 10 from poliomyelitic monkey brains. In addition there were included 1 strain of enterococcus and 1 of hemolytic streptococcus—the latter being recovered from the air.

Cultural Characteristics.—With few exceptions, the non-hemolytic streptococci produced on rabbit blood agar small, round, greenish tinged colonies with a very narrow zone of clearing; exceptional colonies did not produce this zone. On rabbit blood dextrose agar the colonies precipitated the medium to dark brown, just underneath and surrounding the colony. In fluid medium, consisting of chopped meat broth, ascitic dextrose broth, dextrose and plain broth, all the streptococci grew profusely within 18 to 48 hours. The dextrose media showed a granular appearance along the sides and a heavy, whitish precipitate; the other media revealed either granular or diffuse, but also luxuriant growths.

Morphologically long chained forms predominated but 2 of Rosenow's cultures and some selected streptococci obtained from the air and from other non-poliomyelitic sources revealed diplococcus forms often in short chains. All showed pleomorphism, and when a long chained form was inoculated into a rabbit, the tissues would usually reveal diplococcus forms; this happened irrespective of the source of the culture.

Tests for fermentation were made on 24 cultures of the non-hemolytic streptococci. Four distinct groups containing cultures having identical reactions could be determined; the reactions of each group as a whole, however, differed from those of the others. A fifth group contained 11 heterogeneous strains which were unrelated to any of the others. All cultures were classified as follows:

Group I. 1 strain of streptococcus obtained from air (Dr. Carrel's room).
          2 strains of Rosenow's series given us by Eli Lilly and Company.
Group II. 1 strain of streptococcus obtained from air (hood).
          1 strain from non-poliomyelitic brain.
          1 strain from poliomyelitic brain.
Group III. 1 strain (No. 349) of Rosenow's series from American Type
          Culture Collection.
          1 strain obtained from Rosenow.

Group IV.   3 strains from poliomyelitic brains.
            2 strains obtained from Rosenow.

Group V.   Heterogeneous. Includes 2 strains obtained from the air, 1
            from Eli Lilly and Company, and 8 from poliomyelitic
            monkey brains. In this group only one culture fermented
            mannitol; 3, salicin; 6, inulin (3 faintly so); 3, raffinose;
            and all fermented dextrose, maltose, lactose, and saccharose.

It is noteworthy that there was no uniformity in fermentation reactions. Furthermore, when 2 or more cultures were recovered from the same brain, the individual growths showed different responses to the tests.

Serological Reactions.—With regard to direct and cross-agglutination reactions with rabbit immune serum, the different cultures of non-hemolytic streptococci, irrespective of source, also showed a marked heterogenicity.

Rabbits were immunized by the method of injecting intravenously first dead then living cultures. 3 to 4 inoculations were given at daily intervals and after a rest of 4 to 5 days, the daily series was continued, proceeding thus for over a month. 10 days after the last injection, the rabbits were exsanguinated. The blood serum then revealed agglutinins in dilutions of 1:320 to 1:5120 to homologous strains. In only one instance was the titre 1:40.

As antigens for the preparation of the rabbit serum, 10 strains were used, of which 7 were directly or indirectly obtained from Rosenow and were classified by him as "poliomyelitic" streptococci; 2 were recovered from cultures of "ground" broth or chopped meat medium;[13] and 1 was derived from cultures of poliomyelitic monkey brain. Agglutination tests were set up after the manner of Rosenow with the 10 sera and 25 different cultures. The latter included an enterococcus, a *Micrococcus tetragenus*, a hemolytic streptococcus, and 22 strains of non-hemolytic streptococci derived from the air, from media, from non-poliomyelitic guinea pig and monkey brains, and from poliomyelitic monkey brains.

A summary of the results reveals that 3 of the sera prepared with 3 cultures, 2 of which were derived respectively from "ground" media and 1 from a poliomyelitic monkey brain, agglutinated only the homologous cultures. The remaining 7 sera agglutinated, apart from the homologous strains, very few of the 25 cultures put to test. The latter 7 sera were all prepared with different strains ultimately derived from Rosenow. The positive results with these sera follow:

Serum 809, positive only with 2 strains of streptococci obtained from poliomyelitic monkey brains, in dilutions of 1:640 and 1:2560; and Serum 866, positive only with 1 similar strain (1:160). Serum 2254, positive only with 2 other Rosenow strains (1:160 and 1:320). Thus also Serum 349 (1:160 and

1:1280). Serum 3002, positive only with 1 other Rosenow strain (1:40); similar to this was Serum 3007 (1:1280). Serum 3005, positive only with 1 other Rosenow serum (1:320) and with a non-hemolytic streptococcus obtained from ground normal guinea pig brain.

One may therefore conclude that from the view-point of serological reactions the non-hemolytic streptococci, whether obtained from poliomyelitic or other material, form a class of bacteria of dissimilar antigenic function. Furthermore, pooled serum from monkeys recovered from experimental poliomyelitis (and similar serum from normal monkeys, employed as control) failed to agglutinate any of the non-hemolytic streptococci, including Rosenow's 7 strains. Finally, a similar heterogenicity among the poliomyelitic and among the non-poliomyelitic streptococci was revealed by precipitin tests.

Skin Reactions in Rabbits.—No skin reactions were noted in rabbits when the animals were injected intracutaneously with a filtrate from Hartley's modification of Douglas' tryptic digest broth[21] inoculated with Rosenow's strains of streptococci and incubated for 10 days, nor were skin reactions visible with lysates of these microorganisms prepared *in vivo* in immunized rabbits.

Pathogenicity for Rabbits.—It has been stated by Rosenow and others that rabbits dying of streptococcus infection showed signs and lesions identical with those of poliomyelitis in man and monkeys.[1,2,4]

36 rabbits were inoculated intracerebrally with 0.35 cc. to 0.4 cc. of 18 to 24 hour old broth cultures of non-hemolytic streptococci derived from the air, from non-poliomyelitic and poliomyelitic monkey brains. 18 different cultures were used and each was inoculated into 2 rabbits. The results are tabulated on page 446.

In spite of the fact that the filtrable virus of poliomyelitis cannot be successfully implanted on the nervous tissue of rabbits,[26] the streptococci, on the other hand, readily induced a reaction which is not characteristic of the virus of poliomyelitis but is typical of streptococcus infection. It is to be noted that the microorganisms derived from poliomyelitic and non-poliomyelitic tissues; or from the air; or, as has been previously reported,[13] from "ground" medium, and from ground normal or herpes virus-infected brain, show no distinctive effects in rabbits. Many of the cultures of streptococci,

[26] Flexner, S., and Lewis. P. A., *J. Am. Med. Assn.*, 1910, liv, 45.

obtained from different sources, are pathogenic for rabbits, producing purulent meningoencephalitis complicated by streptococcic septicemia, and few of these bacteria are apparently non-pathogenic. It is noteworthy that some rabbits are resistant to pathogenic strains —an observation also made in the studies on herpes virus encephalitis.[13]

Strain	Source	Results in each of 2 rabbits
3002 (a)	Rosenow (from poliomyelitic tissue)	1 died within 24 hrs.; the other survived
3005	" "	Both survived
3007	" "	" died within 24 hrs.
349	" "	1 died within 24 hrs.; the other survived
809	" "	Both died within 24 hrs.
866	" "	" " " " "
2254	" "	" survived
1	Experimental poliomyelitis of monkey	" died within 48 hrs.
3	" "	1 died within 48 hrs.; the other survived
6	" "	" "
7	" "	Both died within 24 hrs.
15	" "	" survived
10	" "	" died within 24 hrs.
10E5	" "	1 died on the 5th day; the other survived
8	Non-poliomyelitic monkey	Both died within 24 hrs.
Air C	Air Dr. Carrel's room	" " " " "
Air H	Air routine laboratory	" survived
Air H 2	" " "	1 died within 24 hrs.; the other survived

(a) Strains 3002, 3005, 3007 were obtained from Eli Lilly and Company; 349, from the American Type Culture Collection; and 809, 866, and 2254 directly from Dr. Rosenow.

The rabbits that died exhibited purulent meningoencephalitis. Their brains, and in most instances the heart's blood, yielded pure growths of streptococci. In respect to the action of streptococci in rabbits, we have confirmed in detail the previous findings of Bull,[6] to whose work the reader is referred for a comprehensive description of the signs and histopathology in injected animals. These experiments are also in complete agreement with those reported by Olitsky and Long on the rôle of streptococci in herpes virus encephalitis,[13] and the reader is also referred to this paper for a fuller account of the action of streptococci in rabbits.

DISCUSSION AND SUMMARY.

It is the opinion of Bull,[6] that the streptococci recovered from poliomyelitic tissues, while having no etiological or pathological relationship to the virus of poliomyelitis, occur as secondary invaders in the disease. Smillie[7] and Amoss[8] indicated that the bacteria may be agonal invaders.

The results of the experiments reported in this paper point to another source of the streptococci. They occur as contaminants which are introduced into the cultures during the process of grinding tissues. The source of the streptococcus may therefore be the air of the place in which the cultures are made. We have come to this conclusion because first, the tissues of which cultures yielded streptococci were derived from a number of monkeys with experimental poliomyelitis still in a vigorous state. Secondly, when the tissues were ground bacteria were noted much more frequently in their cultures than in those in which fragments of the same brains were used. Thirdly, microorganisms occurred more often in cultures made in the routine laboratory than in a special room where asepsis was carried to the extreme of a major surgical operation on man. Fourthly, streptococci were obtained from the air of the places where cultures were made. Finally, there is no correlation between the cultures of two portions of the same brain.

The streptococci occurred in some cultures in pure growth and in others admixed with other ordinary species of bacteria. The latter were often found, in turn, in pure culture and what applies to streptococci, as mentioned in the preceding paragraph, applies equally to the staphylococci, diphtheroids, spore-bearing rods, and other miscellaneous, familiar microorganisms.

We could not determine that there exists any etiological relation of the streptococci to poliomyelitis. The fermentation reactions of the microorganisms obtained from the air, from non-poliomyelitic and poliomyelitic monkey brains indicate that bacteria from any of these sources are markedly different. So also with the serological reactions of agglutination and precipitation. Furthermore no agglutination was observed when the serum of monkeys convalescent from experimental poliomyelitis was mixed with any of the streptococci

recovered or those received directly or indirectly from Rosenow. Moreover, the intracerebral injection with cultures, irrespective of their source, induced in rabbits a purulent type of meningoencephalitis, often associated with streptococcic septicemia. This result is at marked variance with any known effects of the true filtrable virus of poliomyelitis in man and in the monkey.

VIRUS NEUTRALIZATION EXPERIMENTS WITH ROSE-NOW'S AND PETTIT'S ANTIPOLIOMYELITIC SERA.

By FRED W. STEWART, M.D., and PETER HASELBAUER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

During the past decade three types of antipoliomyelitic sera have been employed in the therapy of acute anterior poliomyelitis. These are, first, the sera of convalescent human poliomyelitis; second, sera from horses immunized against the streptococci supposed by Rosenow and others (1–12) to be etiologically related to poliomyelitis; third, the Pettit serum prepared at the Pasteur Institute (13). The last mentioned product consists of sheep serum or horse serum from animals supposedly immunized against poliomyelitis virus through repeated intravenous injections of emulsions of spinal cords of poliomyelitic monkeys. The experimental evidence upon which Rosenow and his coworkers base their claims for the efficacy of antistreptococcus poliomyelitis serum is discussed in another paper (14) and need not receive further mention at this moment. As regards the antipoliomyelitic serum of Pettit, it is sufficient to say that, so far as can be determined, the experimental evidence for its therapeutic value rests upon a single neutralization experiment of somewhat doubtful significance, as we shall later show. Since both the Rosenow and Pettit's serums have been more or less widely employed, it was deemed desirable to study this neutralizing action on the virus of poliomyelitis.

Amoss and Eberson (20) have already made a series of neutralizing tests with the Rosenow serum and failed to detect neutralizing properties. The Pettit serum was originally prepared from sheep "immunized" by intravenous injection with suspensions of poliomyelitic monkey cords. Later, "immune" horse serum was produced by a similar procedure (15). As previously mentioned, only one neutralization experiment is recorded. Two monkeys, A and B, received intracerebrally virus plus "immune" sheep serum and virus alone respectively. The monkey with the serum-virus mixture remained normal, but the monkey receiving virus

alone developed typical poliomyelitis. Since occasional neutralization of poliomyelitis virus by sheep sera is not at all unknown in this laboratory (21), we were inclined to suspect that the apparent favorable result obtained by Pettit rested upon some such chance neutralization; this was more probable since in the experiment reported the control neutralization set up was made with virus alone instead of with a mixture of virus plus "normal" sheep serum. This is an unsafe procedure in testing a heterologous serum. For this occasional virus neutralization by sera of non-susceptible animals, no immediate explanation is apparent.

TABLE I.

Series I.

Monkey	Intracerebral inoculum	Result*
1	Pettit antipoliomyelitis serum............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
2	Rosenow antipoliomyelitis serum concentrated (Eli Lilly)....................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
3	Human convalescent serum *a*.............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
4	Human convalescent serum *b*.............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
5	Normal horse serum..................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
6	Normal human serum.................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Abortive poliomyelitis

* Confirmed by histological study.

Numerous clinical papers (15–19) are available, showing the supposed beneficial effect of the Pettit serum in the arrest of human poliomyelitis. In none is it possible for an unbiased reader to ascertain any certain evidence that the beneficial results attained were really due to the serum therapy. In fact, certain favorable results are claimed in late paralyzed cases well beyond the period of acute disease and even in cases of myelitis certainly clinically of non-poliomyelitic variety.

The experiments to be reported in the present paper are several. All were rigorously controlled.

Macacus rhesus monkeys were used as experimental animals. The poliomyelitis virus serving for infection was the Rockefeller Institute M.A. strain. The Rosenow serum was obtained from Eli Lilly and Company, the Pettit serum through the kindness of Dr. A. Pettit; a similar serum was later prepared by us in accordance with Pettit's original published method. As control sera we employed those of convalescent humans, convalescent monkeys, normal monkeys, normal horse, and normal sheep. The sera to be tested were mixed with either suspensions or Berkefeld filtrates of fresh virus, were incubated 2 hours, and allowed to stand overnight in the ice box. All animals were etherized and infected

TABLE II.

Series II.

Monkey	Intracerebral inoculum	Result
7	Pettit antipoliomyelitis serum............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
8	Rosenow antipoliomyelitis serum unconcentrated (Eli Lilly)...................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
9	Human convalescent serum................ 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
10	Monkey convalescent serum............... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Remained well
11	Normal horse serum...................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
12	Normal monkey serum.................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis

intracerebrally. The results are best shown by Tables I to VI. We are concerned only with the development of definite paralytic poliomyelitis and not with the question of death or survival, degree of paralyses, or length of incubation periods. In small series these are deemed of doubtful significance.

It is evident from the three series of experiments summarized in Tables I to III that no virus neutralizations have occurred with Rosenow's serum—at least with the three different samples employed. The Pettit serum has neutralized, but the neutralization was inconstant. To test further this serum, the virus used was prepared as a

suspension rather than a Berkefeld filtrate. Pettit utilized suspensions in his own neutralization tests. Further results with both suspension and filtrate appear in Table IV. It is quite apparent that the Pettit serum does not consistently neutralize virus.

TABLE III.

Series III.

Monkey	Intracerebral inoculum	Result
13	Rosenow antipoliomyelitis serum........... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
14	Pettit antipoliomyelitis serum............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Questionable poliomyelitis
15	Normal horse serum..................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis

TABLE IV.

Series IV.

Monkey	Intracerebral inoculum	Result
16	Pettit antipoliomyelitis serum............. 0.9 cc. 5% suspension poliomyelitis virus......... 0.3 cc.	Typical poliomyelitis
17	Pettit antipoliomyelitis serum............. 0.9 cc. 5% Berkefeld filtrate poliomelitis virus..... 0.3 cc.	Questionable symptoms only. No paralysis
18	Pettit antipoliomyelitis serum............. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Typical poliomyelitis
19	Normal horse serum..................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus.... 0.3 cc.	Poliomyelitis (excitement, fatigue, prominent facial paralysis only)

Following these experiments, we determined to prepare sheep sera according to the method of Pettit. Three sheep were used. Previous to the first inoculation, the sera of these animals were tested for virus neutralization in monkeys. The results appear in Table V.

TABLE V.

Series V.

Monkey	Intracerebral inoculum	Result
20	Normal serum Sheep I.................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1965 and 1983............................. 0.3 cc.	Remained well
21	Normal serum Sheep I.................... 0.9 cc. 5% suspension poliomyelitis virus 1993..... 0.3 cc.	Typical poliomyelitis
22	Normal serum Sheep II................... 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1965 and 1983........................ 0.3 cc.	Typical poliomyelitis
23	Normal serum Sheep III.................. 0.9 cc. 5% Berkefeld filtrate poliomyelitis virus 1993................................ 0.3 cc.	Remained well
24	Normal monkey serum................... 0.9 cc. 5% suspension poliomyelitis virus.......... 0.3 cc.	Typical poliomyelitis

TABLE VI.

Series VI.

Monkey	Intracerebral inoculum	Result
25	Normal serum Sheep II................... 0.9 cc. 5% suspension poliomyelitis virus .. .. 0.3 cc.	Remained well
26	"Immune" serum Sheep II................ 0.9 cc. 5% suspension poliomyelitis virus .... 0.3 cc.	Typical poliomyelitis
27	Normal serum Sheep III.................. 0.9 cc. 5% suspension poliomyelitis virus .... 0.5 cc.	Remained well
28	"Immune" serum Sheep III.............. 0 9 cc. 5% suspension poliomyelitis virus ..:......0.5 cc.	Typical poliomyelitis
29	5% suspension poliomyelitis virus .... 0.3 cc.	Typical poliomyelitis

Thus it would appear that Sheep I serum, previous to any immunization, inhibited Berkefeld filtrate once and at another time failed to inhibit suspension; that Sheep II serum failed to inhibit

Berkefeld filtrate; that Sheep III serum inhibited Berkefeld filtrate. Sheep I was lost from intercurrent disease. Sheep II and III were given twenty-one biweekly, intravenous injections, each consisting of 5 cc. of 5 per cent virus suspension from fresh or glycerolated monkey spinal cords. The animals were bled after a brief rest, and the sera were again tested for neutralization or inhibition.

The lack of consistency in results is well shown by the fact that both normal sera inhibited or neutralized virus, whereas sera from the same animals after "immunization" failed to do so.

CONCLUSIONS.

1. The Rosenow antistreptococcic poliomyelitis serum concentrated or unconcentrated does not neutralize the virus of poliomyelitis as tested in monkeys.

2. The Pettit antipoliomyelitis horse serum neutralizes the virus only occasionally.

3. "Immune" sheep sera prepared according to the method of Pettit have not neutralized virus even when the normal sera of the same animal have given neutralization.

4. The reason for such chance neutralizations is obscure and should not be confused with the constant virus-neutralizing action of both human and monkey convalescent sera.

5. Experimental evidence affords no basis for the use of either the Rosenow or the Pettit serum in the therapy of poliomyelitis.

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OF

EXPERIMENTAL MEDICINE

STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

I. The Action of Light on White Blood Cells in Vitro.

By W. R. EARLE.*†

(From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville.)

Plates 9 and 10.

INTRODUCTION.

In the course of some work upon photobiological sensitization, Busck (1) found that, upon exposure of blood cells of the dog, suspended in .85 per cent NaCl solution, to light from a carbon arc, all of the red cells were hemolyzed within 15 minutes, whereas the white cells were to all appearances unchanged (Experiment 27, page 491).

Lewis and Lewis (5), from their work on tissue cultures of blood cells from lower vertebrates, dogfish, skate, certain teleost fishes, the frog, the toad, and the snake, make the following statement. "The blood-cultures from these cold blooded animals were kept at room temperature and out of the strong light, as the latter has a decidedly injurious effect upon the cells" (page 102).

In addition to these two, several other references may be cited as of interest. Reed (7), using the carbon-tungsten arc, irradiated the blood of the dog *in vivo*, the light being directed through the wall of a quartz tube inserted in the course of the carotid artery. Directly after the irradiation he found an absolute leucopenia and an absolute lymphopenia, but a relative lymphocytosis.

Miles and Laurens (6), subjected dogs to 1 hour irradiation a day, using a carbon arc which had been so adjusted as to give an intensity of light equal to that of sunlight at Washington, D. C. They found that following irradiation there was a marked rise in the white blood count due to a rise both in the number of neutrophils and of lymphocytes. They also found that in the blood smears

there were many "smudges," as well as some lymphoblasts and myeloblasts. They interpret the "smudges" as indicating a rapid destruction of white blood cells.

Clark (2), irradiating the ears of rabbits with light from an iron arc, found that there was an immediate drop in the total white blood count, and that this drop was due to a lymphopenia. Irradiating similar animals through a glass screen she found the total white cell count unchanged after irradiation, but found an absolute lymphopenia.

In an attempt to follow the changes shown by certain leucocytes in tissue cultures[1] of mammalian blood cells, work was greatly hindered by the exceedingly rapid and extreme degeneration of all types of white blood cells in the cultures. In an effort to determine the etiology of this degeneration about 1500 cultures were examined.

Materials and Methods.

The blood from which these cultures were planted was taken from cats, guinea pigs, and rabbits. Most of the work, however, was done on the blood of the rabbit. The general technique used for the preparation of the cultures was as follows. Blood was drawn, either by heart puncture or, after opening the abdomen with the animal under an anesthetic, from the abdominal aorta. It was immediately transferred to oiled or paraffined Pyrex tubes which were stoppered and centrifugated. When the blood clot had formed it was removed from the tube *in toto*, washed in Tyrode solution (pH 7.4), and the buffy coat of the clot was cut up in the same solution, by means of a pair of iridectomy scissors. Fragments obtained in this way were transferred to "non-corrosive" cover-glasses[2] by means of a platinum loop and were then covered by a drop of some one of the following media: Tyrode solution, autogenous serum, autogenous plasma, autogenous heparin plasma, or mixtures of these with chick embryo juice. The cover-glasses were inverted over hollow ground slides of the same glass, and the edges were sealed with "Salvoline," a high melting point petroleum jelly. This whole process was carried out aseptically, and usually required not longer than 1 hour for its completion. These cultures were placed in trays, in groups of five, and set in a dark, water-jacketed incubater which was kept at 37.5°. For examination, a whole tray of slides was removed and set in the microscope hot box which was also kept at 37.5°. Slides were there changed from the tray to the microscope stage as desired.

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[1] The term "culture" is applied to these preparations throughout this whole work, although it is realized that many of the preparations are not true cultures in the sense that they show active growth.

[2] These cover-glasses and slides were secured from Arthur H. Thomas Company.

In the preliminary work, several different microscope light sources were employed. These were all incandescent, tungsten filament, vacuum- or gas-filled, electric globes. They ranged in current consumption from 15 to 200 watts and operated at from 6 to 120 volts. The spectra of two of these sources are shown in Spectra 2 and 3, Fig. 4. These light sources were used either with or without auxiliary glass lenses, and were placed approximately 250 mm. from the concave mirror of the microscope. The light was reflected by this mirror, passed up through the three lenses (1.4 N.A.), aplanatic substage condenser (glass), the hollow ground slide, and the chamber of the slide, thus finally reaching the culture.

EXPERIMENTAL.

An outline of the nature and sequence of the degenerative changes seen in these cultures is presented at this point.

A short time after the microscopic examination of any one of the cultures was begun, it was noted that the neutrophils of that culture rapidly became increasingly ameboid, while the flow of cytoplasm within the cells also increased both in extent and in velocity. At the same time, the cytoplasm became more fluid, as was shown by exaggerated brownian movement of the cell granules. Soon cellular movement ceased and the cells became spherical, although for some time, many of them continued to put out slender cytoplasmic processes. The polymorphic nuclei became swollen and rounded (Fig. 1). As a result of this change in nuclear shape, it was difficult to estimate further changes in the volume of the nuclei, but in many preparations it appeared to undergo reduction after a short time. There was neither shrinkage nor collapse of the nucleus however, until very late in the process of degeneration; rather it remained very turgid. The cells gradually became tremendously swollen; in several instances an increase to three times the normal diameter was observed (Fig. 1). It is of interest to note that this indicates an increase to twenty-seven times the normal volume. During this swelling process the viscosity of the cytoplasm continued to decrease. Further, a number of minute oil droplets, which stained with Sudan III, became visible throughout the cytoplasm. These droplets apparently originated from the cytoplasm.

From this stage on, two terminations of the degenerative process were observed. In the first of these, the cytoplasm gradually coagulated around the nucleus, so that the cell membrane was left as a shell which was slowly autolyzed. In the second, the swelling became so great as to rupture the cell membrane, and a jet of cytoplasmic fluid was thrown out into the surrounding medium. At the moment of rupture the dancing granules and oil droplets rapidly agglutinated, evidently from contact with the surrounding medium. The extruded cytoplasm almost instantly coagulated. Subsequently a process of gradual, and more or less complete autolysis of the dead cell began.

When neutral red or Janus green was added to a culture in any of these terminal

stages, it was found that the cells had completely lost their characteristic vital staining with these dyes. The neutrophilic granules did not show their usual light tan color with neutral red, while, unless the cells were too far degenerate, the nuclei took up the dyes. This reaction is characteristic of dying cells.

The type of degeneration shown by the eosinophil appeared very similar to that shown by the neutrophil. There was the same preliminary acceleration of cellular movement and of intracellular cytoplasmic movement. At the same time, the brownian movement within the cell became exaggerated and the cell began to round up. The brownian movement continued and increased for some time after this, but its amplitude never reached that shown by the granules of the neutrophil. This was due, in part at least, to the large number of granules within the cell, and also to their size.

The eosinophil also became swollen, but even in its final stages its diameter rarely exceeded one and one-half times the diameter of the normal cell. It showed nothing like the amount of swelling shown by the neutrophil, nor was it ever observed to increase in size to the point of rupture of the cell membrane. The nucleus of the eosinophil also increased in size, although this increase, also, was less marked than that noted in the nucleus of the neutrophil. As a result of the swelling, this nucleus likewise had a tendency to become spherical, but even in the extreme stages of its degeneration the two lobes could generally be made out.

The cytoplasm gradually coagulated around the nucleus, and, as a result, one side of the cell was often left almost free of granular material. During this process of coagulation and the still later stages of degeneration, the eosinophilic granules slowly lost their affinity for neutral red, while their outlines became hazy and sometimes completely disappeared. When this occurred the whole group of granules was left as an apparently free, but coagulated, conglomerate mass. In some cultures these cells remained in this condition for several days. Oil droplets were observed far more rarely in the eosinophil than in the neutrophil, and even in the one or two instances where they were noted, they were very small.

The degeneration of the basophil was essentially like that of the eosinophil, except that the nucleus became enlarged until it was practically spherical, and, since it increased in size far more rapidly than did the volume of the cell, it often forced aside the cytoplasmic granules until they were all crowded within a very small segment of the cell. Only very small fat droplets were observed in the basophil, and these but once or twice.

The monocyte underwent a series of changes almost identical with those shown by the above cells. Within a short time after examination was begun there was a marked increase in the refractive index of the nucleus, this body becoming distinctly visible. As the brownian movement of the cell vacuoles increased, the cell gradually rounded up and its outline became more clearly visible. At the same time, the cell swelled to some degree, and, as indicated by the increased amplitude of brownian movement within the cell, the viscosity of the cytoplasm decreased rapidly. Coagulation later took place. More fatty material was

liberated within the monocyte during its degeneration than within any other type of cell. All of this fatty material, as in the case of that contained within the polymorphonuclear neutrophil, stained deeply in Sudan III.

Because of the difficulty experienced in finding and identifying the large lymphocyte, its degenerative cycle was not worked out. The small and intermediate lymphocytes, however, were more easily followed. These cells were normally almost motionless and no preliminary stimulation, such as was seen in the degenerative cycle of the other cells, was observed. This, however, does not necessarily mean that such does not exist; more probably it means that, in view of the very low motility of the cell and its small area of cytoplasm, our methods for determining stimulation have not been sufficiently sensitive.

The first visible change in the lymphocyte was a marked increase in the size of the cell, often to one and one-fourth to two times its original diameter. The mitochondria, whether examined unstained or stained by Janus green, swelled somewhat, and then gradually disappeared. There was little change in the nuclear size, but the cytoplasm of the cell became typically edematous. In some cases, the cell wall remained for several days as a distinct turgid shell. No fat droplets were observed in these cells.

In connection with the preliminary studies upon which the preceding description is based, several characteristics peculiar to the distribution of degenerate cultures through any one series gave evidence that the degeneration must have been caused by some factor intimately associated with the process of examination. An attempt was next made to examine the different factors which might be responsible for this degeneration. By substitution of a dry objective for the oil immersion objective, the immersion oil was definitely eliminated as the cause of degeneration. Further, by very careful handling of the culture, the factor of mechanical disturbance, consequent to the process of transfer from the incubator to the hot box, and from the tray to the microscope stage, was also ruled out. The temperature of the microscope hot box was next readjusted, being set at 39° (±.1), the blood temperature of the rabbit and the chick. Despite these precautions the number of degenerate cultures was not materially decreased, while the peculiar type of cellular degeneration remained unchanged.

The elimination of these factors, together with the data concerning the distribution of degeneration through any one series of cultures, strongly indicated that the action of light, ultra-violet, visible, or infra-red, either from the surrounding daylight or from the micro-

scope lamp itself, was the most probable agent in causing the degeneration. In order to test out the possibility that light was the causative factor, three series of twenty cultures were prepared. These cultures were inoculated in very dim daylight, and were placed in the dark incubator as quickly as possible. All further handling of these cultures was also done only in very dim light. The individual cultures were taken from the incubator only as needed, placed on the microscope stage with the lamp cut off, and were allowed to remain in the dark for from 5 to 15 minutes in order that the cells might recover from any slight abnormalities due to shaking. Protocols of a group of representative cultures taken from these three series are presented below.

Experiment 1.

Culture 546.—Blood was drawn from a young rabbit under light, ether anesthesia. Cultures were planted in autogenous serum and incubated at 39°. The light source used in the examination of these cultures was the same as for Culture 270. *13.5 hours after inoculation:* Examination and irradiation of the culture were begun. The culture at this time was normal; the cells were moving around slowly. The first observations were made with the substage iris diaphragm closed to its limit, and the light reaching the culture further reduced by means of a Wratten and Wainwright Filter 58 (green). After several minutes examination, the iris diaphragm was opened, but the filter was left in place. The neutrophils almost immediately began moving more rapidly. The green filter was next removed, thus increasing the intensity of light reaching the culture. Brownian movement of the cell granules of the neutrophils began within 10 seconds, and soon became greatly augmented. Within 1 minute cell motion itself had also greatly increased. 1 minute later the filter was again inserted and the examination continued with closed iris diaphragm for 2 minutes. During this time cell movement became slowly reduced, both in velocity and in magnitude. At the end of this time the light was cut off. 1 minute later it was turned on again; during this interval in the dark cell movement had practically ceased, but within a minute or so after the light was turned on it was back to the level at which it was observed at the beginning of the examination. The light was again cut off, this time for 10 minutes, then cut on again. At this time the cells were badly swollen, were rounded up, and cell movement had ceased. The nuclei of the cells were spherical and edematous. There was also a decided increase in the brownian movement of the cell granules. The total time of examination and irradiation was less than 20 minutes.

Cultures 582 and 583.—Blood was taken from the abdominal aorta of a young rabbit under light, ether anesthesia. Cultures were inoculated in autogenous

serum and incubated at 39°. The light source used in the examination of the culture was a 60 watt, 110 volt, vacuum-filled globe. 35 mm. of water was inserted into the optical path. For the spectrum of this light source see Spectrum 2, Fig. 4. *90 minutes after inoculation:* These two cultures were taken from the dark incubator and placed side by side on the microscope stage, in such a manner that while Culture 582 received the full intensity of light coming up through the microscope condenser, Culture 583 rested on the black microscope stage and was protected by it from exposure to light. Examination and irradiation were begun. At this time the cells of Culture 582 appeared perfectly normal. The neutrophils were very active, and there was no sign of rounding up. *10 minutes later:* These cells were still very active; if any change had occurred, there was a slight stimulation. *30 minutes:* All of the neutrophils of Culture 582 were rounded up; their nuclei were slightly swollen, and their cytoplasm had coagulated, while their granules appeared somewhat hazy. At this time Culture 583 was given its first examination. The cells of this culture were perfectly normal; the neutrophils were moving around actively. No sign of the degeneration noted in Culture 582 could be seen.

The results shown by the cultures examined in this experiment may be briefly summarized as follows.

1. Radiant energy of such intensities and wave-lengths as originated from the microscope light source, acting on these cultures for periods of time varying from 2 minutes to about 70 minutes, produced an extreme and unmistakable effect on the cultures.

2. This action may be said to have had three phases, as follows. (a) There was first a short latent period during which no effect was noted. This period lasted from 3 seconds to 30 minutes. (b) Following the latent period there was an increase in the activity of the cell; the cellular movements were more extensive and more rapid. There was also a great decrease in the viscosity of the cytoplasm, as indicated by the tremendously increased amplitude of brownian movement of the granules within the cell. (c) Following this, there was a terminal period during which the cell rounded up. The viscosity of its cytoplasm still further decreased. This was accompanied by a swelling of the cell, or often, by coagulation.

3. The degeneration noted in the cells of this series of cultures, apparently coincided in every respect with the degeneration noted in previous cultures and described as of unknown etiology.

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scope lamp itself, was the most probable agent in causing the degeneration. In order to test out the possibility that light was the causative factor, three series of twenty cultures were prepared. These cultures were inoculated in very dim daylight, and were placed in the dark incubator as quickly as possible. All further handling of these cultures was also done only in very dim light. The individual cultures were taken from the incubator only as needed, placed on the microscope stage with the lamp cut off, and were allowed to remain in the dark for from 5 to 15 minutes in order that the cells might recover from any slight abnormalities due to shaking. Protocols of a group of representative cultures taken from these three series are presented below.

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2. This action may be said to have had three phases, as follows. (a) There was first a short latent period during which no effect was noted. This period lasted from 3 seconds to 30 minutes. (b) Following the latent period there was an increase in the activity of the cell; the cellular movements were more extensive and more rapid. There was also a great decrease in the viscosity of the cytoplasm, as indicated by the tremendously increased amplitude of brownian movement of the granules within the cell. (c) Following this, there was a terminal period during which the cell rounded up. The viscosity of its cytoplasm still further decreased. This was accompanied by a swelling of the cell, or often, by coagulation.

3. The degeneration noted in the cells of this series of cultures, apparently coincided in every respect with the degeneration noted in previous cultures and described as of unknown etiology.

4. The continued action of light was not essential for the completion of the degenerative process in the cell. A very short exposure (3

minutes), produced little or no visible effect at the time, but even this was often found to be sufficient to cause the death of the cells in the culture within a few hours.

5. There was a curious irregularity in the time required for the degeneration of different cultures from the same series even when all were irradiated by the same source of light. For instance, the degeneration time of one culture was 35 minutes, while that of another in the same series was 90 minutes.

The positive findings summarized above have been confirmed by several hundred other cultures, examined at a later time in the accumulation of other data concerning this degeneration.

It having been definitely determined that irradiation of the cultures did cause a rapid degeneration of the white blood cells, an attempt was made to determine what wave-lengths of light caused this degeneration. The degeneration had been constant in its appearance upon exposure of the culture to light from any of the previously described sources. As a standard source of light for the further analysis of the problem, the 6 volt, 108 watt microscope lamp was chosen (Spectrum 3, Fig. 4). Besides the condenser system, 2 mm. of Corning daylight glass and a 35 mm. water-cell were also inserted in the path of the light. The light reaching the eye with this arrangement was of nearly daylight quality and of an intensity comfortable for visual work with a binocular microscope fitted with an oil immersion lens and 12 × oculars.

With this light source, and optical system, the degeneration might conceivably have been due to a concentration of infra-red rays on the culture, with a resultant heat coagulation of the cells. In order to investigate this possibility, a special glass "heat filter" (Spencer Lens Company) absorbing a large part of the infra-red rays (65–75 per cent) was also inserted into the optical path (Spectrum 4, Fig. 4).

In spite of the removal of such a large part of the infra-red heat waves from the light reaching the culture, the degeneration produced was apparently exactly the same as if no heat filter or water-cell had been used. While it is conceivable that this degeneration might have resulted from the residual infra-red waves reaching the culture through the optical system, the fact that there was no change in the rate of degeneration of the cells after the elimination of most of these

infra-red wave-lengths, makes it appear conclusive that the effect produced was not due to a heat coagulation of the cell.

With this possibility eliminated, the next step was to determine what range of wave-lengths was responsible for the degeneration. In order to do this the same light source and optical system were used, while various Wratten light filters were substituted for the Corning daylight glass. By this means the spectral range of the light admitted to the cultures was divided into several wave-length zones. The filters employed, together with their transmission zones and total transmissions (3), are listed in Table I, while spectrograms showing their light transmissions are shown in Spectra 5, 6, 7, Fig. 4.

TABLE I.

Filter		Wave-length zones in $\mu\mu$	Total transmission
Color of transmission	Number		
			per cent
Blue	45	320 to 390; 430 to 550; infra-red. (Spectrum 5, Fig. 4.)	5.0
Green	58	475 to 630; 690 to infra-red. (Spectrum 6, Fig. 4.)	23.0
Red	29	600 to infra-red. (Spectrum 7, Fig. 4.)	6.6

The results of this experiment showed that light transmitted by any one of these filters was active in causing the degeneration noted. Further, it was found that the time periods required for the degeneration of different cultures varied over a wide range for any one filter, and that the time periods required for the degeneration of different cultures of the same series, varied through the same wide limits when any one of the three filters, or when no filter was employed.

Hanging Drop Cultures of Whole Blood.

While the preceding experiments have definitely shown that the action of radiant energy did cause an extreme degeneration of the leucocytes, it was recognized that cells in this type of culture were under very abnormal conditions. They had been subjected to

centrifugation and lowering of temperature to 22°C. for about an hour, then washed with an artificial saline solution, and planted in an artificial culture medium. Any one or all of these manipulations might make these cells particularly susceptible to the action of radiant energy, either by increasing the fragility of the cells, or by otherwise changing their physical or chemical characteristics. In order to control as many of these factors as possible, another group of cultures was prepared and examined. In the preparation of these cultures the following technique was employed.

A drop of blood was drawn from a vein of a rabbit's ear, a cover-slip quickly touched to it, and then given a slight jerk so that the drop was spread out into a streak on the cover-slip. The cover-slip was then inverted on a hollow ground slide, and the edges of the cover-slip were sealed with "Salvoline." The culture was immediately placed at blood temperature and irradiation was begun within 10 minutes. These cultures were prepared in groups of three or four. Of these, one was irradiated while the other two or three were kept in the dark as controls.

The protocol of a representative culture of this series, together with its control, is presented below.

Culture 1062.—Examination and irradiation begun. The neutrophils at this time were moving around actively; the other cells appeared normal. *30 minutes later:* The neutrophils were moving around very rapidly as though markedly stimulated. *67 minutes:* The neutrophils had practically ceased moving around and were rounding up. *100 minutes:* The neutrophils within the illuminated area of the culture had all coagulated.

Culture 1063.—Control culture, kept in the dark. *200 minutes after inoculation:* Examination begun: The neutrophils were normal and were moving around fairly rapidly.

As may be seen from this experiment, the degeneration observed in saline and serum cultures of white blood cells appeared in a perfectly characteristic manner in these hanging drop preparations of whole blood. Since this was true, it appeared certain that the degeneration of leucocytes *in vitro* under the action of light, was not dependent upon prolonged temperature changes, the centrifugation to which the cells in culture were subjected, or the relatively rough handling necessary in the preparation of the tissue cultures, nor was it dependent on changes in saline concentration to which the cells in culture were subjected. It seemed to be a property of the leucocyte as it occurred in freshly drawn blood.

This degeneration of the leucocytes in hanging drop cultures of whole blood was also found to occur in perfectly typical manner upon irradiation of the culture through the various light filters previously described. It was also found to occur as usual upon irradiation of the culture through a 6 mm. thickness of Corning "Noviol C" glass, which transmitted only wave-lengths longer than $480\mu\mu$, and a heat filter made up of a 3 cm. thickness of 5 per cent cupric sulfate.

In examining the data accumulated in all of these experiments, however, a tabulation of the time required for the degeneration of

TABLE II.

Irradiation Time Required to Cause Polymorphonuclear Neutrophils to Round Up, in Hanging Drop Cultures and in Hanging Drops of Whole Blood.

In tissue culture		In hanging drops of whole blood	
Culture number	Time	Culture number	Time
	min.		*min.*
472	10	1051	170
507	10	1059	95
587	30	1060	45
588	35	1062	67
589	90	1317	120
608	60	1320	58
612	12	1322	75
614	41	1512	60
618	7	1577	55
631	14	1578	60
Average..........	31		80

the leucocyte, *e.g.* the neutrophil, in ten representative hanging drop preparations of whole blood, and in ten representative tissue cultures prepared from the buffy coat of the centrifugated clot, showed that in the former the average time was 80 minutes, in the latter, only 31 minutes. Such a comparison of the degeneration time of representative cultures of both types is presented in Table II.

DISCUSSION.

From the data presented, it may be seen that the degeneration observed in these cultures of blood cells may be ascribed to the action

of light on the cells.   It is well known that the Röntgen or ultra-violet regions of the spectrum may have a very profound effect on the organism, and the present work indicates that, certainly in the case of the blood cells *in vitro*, this action extends far down into the visual spectrum, certainly lower than 600 double micra.   Further, as in the case of the generally known action of the shorter light waves of the spectrum, the action of these longer light waves is to produce first a stimulation, and later a degeneration of the irradiated cells.   That this process of degeneration was conditioned by more than one factor is indicated by the divergent courses which were sometimes seen in the degeneration of different cells of the same type.   In different cultures, cells were seen in which there was little swelling, but a coagulation of the cytoplasm.   In other cultures the cells died in an expanded or lobulated condition (Fig. 3), while in still others, cells were seen with peculiar pouchings of the nuclear or cell walls which gave evidence that at least one change in structure had been a weakening of these walls.   Nor were these so much the peculiarities of single cells, for generally the cells in any one culture, and occasionally, in any one series of cultures, showed a curious similarity in their degeneration. In emphasizing these divergent types of degeneration, however, it should be remembered that in the great majority of cultures there was a remarkable uniformity in the process (Fig. 1).

There were two terminations commonly seen in the degeneration of the neutrophil, under the influence of light.   In the one, the cell swelled to about three times its normal diameter, sometimes even extruding some of its contents; whereas, in the other process, there was more of a gradual coagulation of the cell.   What the fundamental differences are, which decide the type of degeneration any one neutrophil will undergo, has not been determined.

The final achromatic dead cells resulting from neutrophils which had undergone the coagulative type of terminal degeneration were often indistinguishable from the typical "non-motile" cell described by Sabin (8).   On the other hand, neutrophils which had undergone the hydropsical type of degeneration, produced dead cells which were tremendously swollen.   When the swelling became so great as to rupture the cell however, the collapsed cell often looked like a typical non-motile.

There is a marked similarity between the hydropsical type of degeneration described above, and the type of degeneration described by Kubie and Schultz (4, page 101) for the neutrophil occurring in cerebrospinal fluid. The technique given by these authors raises the question as to whether this degeneration noted by them was not due, in part at least, to the action of light during the handling of the cells. The similarity between the degeneration that they describe and that shown by the neutrophils in the present study may readily be seen by a comparison of their Figs. 11b and 12b with the present Fig. 1.

As was pointed out in the descriptions of the degeneration of the different types of leucocytes under the action of light, one very striking difference between the cells is the amount of fatty material which becomes visible within their cytoplasm during their processes of degeneration. The cell liberating the greatest quantity of this fatty material was the monocyte, while the neutrophil liberated the next greatest quantity. All other cells liberated relatively little, and none has been observed in the lymphocyte. The fundamental causes of this difference have not been indicated.

We are at present unable to explain the difference in the time required for the degeneration of the leucocytes in serum cultures and in hanging drop cultures of whole blood. It is conceivable, however, that although the rougher handling and abnormal solutions in the cultures planted in serum, may not be the factors sensitizing the leucocytes to light, they may, by some increase in the cell fragility, for instance, make the leucocyte more easily killed upon irradiation. Or it is possible that the longer time required for the degeneration of the leucocytes in hanging drops of blood may be solely due to the presence of red blood cells, and this action may be due either to their reducing the actual amount of light reaching the leucocyte, or, more probably, to other physical or chemical properties which the red cell may possess.

In considering what relation this degeneration of leucocytes *in vitro*, under the action of light, may bear to the action of light on these cells *in vivo*, the conditions in the two instances are so dissimilar that comparison is difficult with our present data. *In vitro* the cells are manifestly under artificial conditions; *in vivo* experimental results are

complicated by systemic reactions.   It is significant, however, that in the work of Reed (7), and also of Clark (2), with light from the unfiltered iron arc, irradiation resulted in an immediate drop in the total white cell count.   It is at least possible that in each of these instances irradiation may have resulted in injury to the leucocytes *in vivo* and their subsequent removal from the blood stream by systemic agencies.   That such injury did occur in the work of Miles and Laurens (6) is positively indicated by the fact that these authors report that following irradiation, in addition to marked fluctuations in the white cell count, there were many "smudges" in the blood smears, these indicating a rapid destruction of white cells.

It should be noted however, that upon irradiating animals with light from the iron arc, filtered through glass, Clark found no such drop in the total count as she had found to follow irradiation with unfiltered light.   There was, however, a marked lymphopenia, compensated by an increase in the number of neutrophils.   This source of light, filtered through glass, might be considered as fairly comparable to the source used in the experiments described in this paper, whereas the light used by Reed, by Miles and Laurens, and by Clark, in her experiment with unfiltered light, was very rich in ultra-violet wave-lengths.   As a consequence of these discrepancies, the nature of the relationship between the results reported in this paper and those reported by the above workers, is far from clear.

SUMMARY.

1. An extreme and rapid degeneration which occurred in tissue cultures of leucocytes from the blood of cats, guinea pigs, and rabbits, is described in detail.

2. This degeneration was found to appear in the culture when the cells were planted in any of the culture media tried, some of which were autogenous heparin plasma, autogenous plasma, autogenous serum, Tyrode solution, and mixtures of these with embryo juice.

3. The specific cellular changes which occurred are described for the different leucocytes.   In general, there was first a latent period during which no change could be observed in the cell.   Following this there was a period of stimulation during which the motion of the cell was greatly accelerated.   This second stage has been observed

in all cells except the lymphocyte, in which it may possibly occur to a slight degree. Finally there was the terminal stage, the stage of degeneration, in which the cell rounded up, lost its motility, and either became badly swollen or else underwent a more or less complete coagulation.

4. The factor causing this degeneration was found to be exposure of the culture to light, as, for example, during microscopic examination.

5. By a reduction of the infra-red part of the spectrum, it was indicated that the effect was not due to a heat coagulation of the cells.

6. This degeneration was also found to occur in the complete absence of ultra-violet wave-lengths.

7. Further, it was shown that this degeneration was caused by light which lay within each of the three wave-length zones (1) $430\mu\mu$ to $550\mu\mu$; infra-red; (2) $475\mu\mu$ to $630\mu\mu$; $690\mu\mu$ to infra-red; (3) $600\mu\mu$ to infra-red.

8. No indication was given as to whether all regions of these zones were active in causing the degeneration, or whether the active rays are limited to certain wave-length bands lying within these zones.

9. This degeneration of the leucocytes under the action of light was also found to occur upon irradiation of hanging drops of whole blood. This is interpreted as showing conclusively that the degeneration was not dependent upon the additional factors of centrifugation, continued lowering of temperature, or the presence of abnormal saline solution.

10. It was noted, however, that the leucocytes in hanging drop cultures required a markedly longer time for their degeneration under the action of light than did the leucocytes in cultures prepared from the buffy coat and inoculated in serum. This is considered as possibly due, either to injury to the cell during centrifugation and subsequent handling, or to some action of the red blood cells present in large amounts in the hanging drops of whole blood.

11. In these hanging drop cultures of whole blood degeneration of the leucocytes was also found to occur when the light reaching the culture was first freed from the larger part of its infra-red and from all of its ultra-violet.

12. It was also shown that the same degeneration was produced by wave-lengths of light lying within each of the three wave-length zones defined in Section 6 of this summary.

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EXPLANATION OF PLATES.

PLATE 9.

FIG. 1. Polymorphonuclear neutrophils of cat; from serum culture. × 1000. These cells show a typical degeneration resulting from an 80 minute exposure to light from the 6 volt 108 watt lamp described in the text, the light passing through an aspheric condenser of 6 cm. diameter, 35 mm. of water, and the microscope substage condenser (glass). By a comparison with the eosinophils shown in Fig. 2, which are photographed at the same magnification, and which are only slightly swollen, it may be seen that these neutrophils are swollen to over three

times their normal diameter. The spherical nuclei and oil droplets are easily seen.

Fig. 2. Polymorphonuclear eosinophils of cat; from serum culture. × 1000. Irradiated for 30 minutes by light from the same light source. Note the swollen nuclei and the tendency for the nuclei to become spherical. The cells are swollen to about one and one-fourth times their natural size.

Fig. 3. Polymorphonuclear neutrophils of rabbit; from hanging drop of whole blood. × 1000. Irradiated for 4 hours with the above light source, the light also passing through 2 mm. of Corning daylight glass, 20 mm. of plate glass, and the infra-red filter described in the text. Note that in this slide the cells have coagulated and died in a partially expanded condition, and that they show little swelling. These cells showed little change in the 2 hours preceding photographing.

PLATE 10.

Fig. 4. Spectra of different light sources used for the irradiation of cells. (1) Spectrum of mercury arc, for comparison. (2) 60 watt, 110 volt, tungsten filament, vacuum-filled, electric globe. Light passing through 35 mm. water, 250 mm. air, glass substage condenser. (3) 108 watt, 6 volt, tungsten filament, gas-filled electric globe. Light passing through a 6 cm. aspheric glass condenser about 1 cm. thick. (4) As in 3, except that light also passed through 35 mm. of water, the special heat filter described in the text, 20 mm. lime-soda glass, 2 mm Corning daylight glass, and the microscope substage condenser (glass). (5) As in 4, except that Wratten Filter 45 was substituted for the daylight glass. (6) As in 4, except that Wratten Filter 58 was substituted for the daylight glass. (7) As in 4, except that Wratten Filter 29 was substituted for the daylight glass.
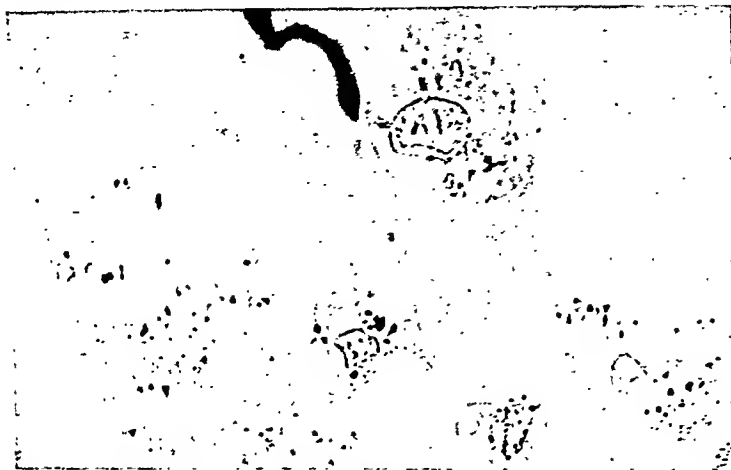
FIG. 1.

FIG. 2.
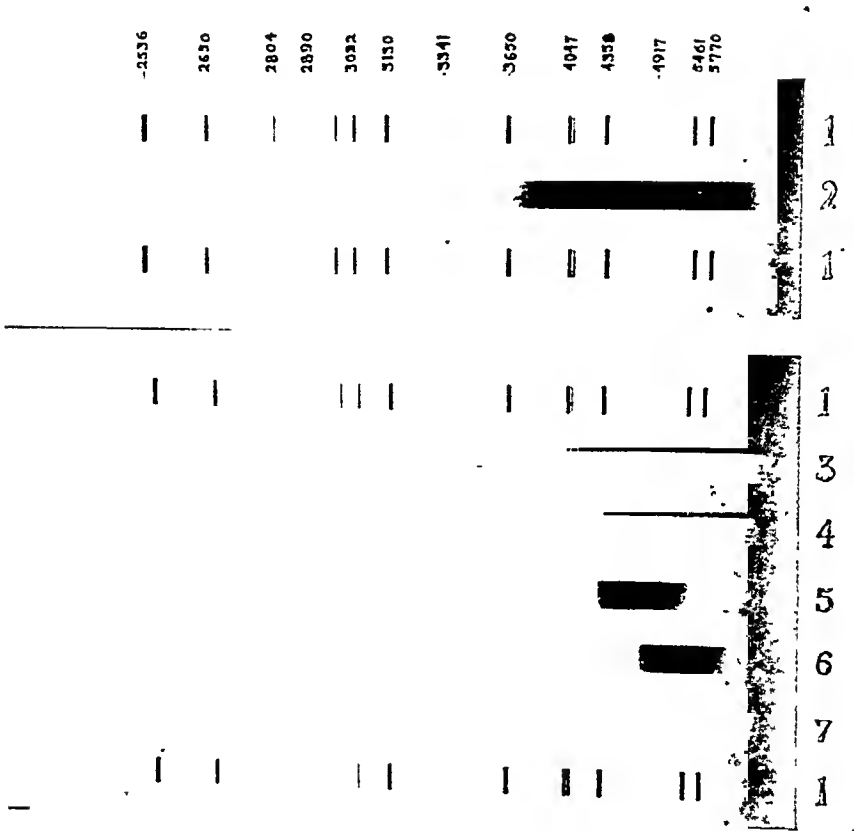
FIG. 4.

THE TRANSFER OF RAT ANEMIA TO NORMAL ANIMALS.

By WILLIAM W. FORD, M.D., and CALISTA P. ELIOT, Sc.D.

(From the Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore.)

PLATE 11.

It has previously been shown by Lauda (1) that following extirpation of the spleen rats develop a severe anemia, characterized by destruction of red blood cells, hemoglobinurea, leucocytosis and an increase of blood platelets. This condition Lauda regarded as infectious and due to some type of virus, which might be vegetating on the mucous membrane of the stomach or intestine and be held in abeyance by the spleen. When the spleen is removed the virus gains entrance into the body and after an incubation period of a few days attacks the blood corpuscles and produces the anemia, the leucocytosis being a response to the infection. Lauda was unable to reproduce this anemia by the transfer of blood or organs to normal animals (except in one instance) but was able to produce it in rats from which the spleen had been removed and which had not yet developed the anemia or had recovered from it. Lauda's observations on rat anemia were subsequently confirmed by Mayer (2) and his associates who further noted that the red blood corpuscles of the anemic splenectomized rat contain small bacilliform bodies which resemble the bodies previously seen by Mayer (3) in the blood of rats with experimental trypanosomiasis and treated with Bayer 205. These bodies Mayer had named *Bartonella muris ratti* because of their resemblance to *Bartonella bacilliformis*, the etiological agent of Oroya fever. Mayer was unable to produce anemia in normal rats with blood containing *Bartonella muris* from anemic rats or to demonstrate *Bartonella muris* in the blood of the injected normal animals. Mayer (4) then observed that *Bartonella muris* could be eliminated from the blood of splenectomized rats by treatment with various compounds of arsenic. Injections of bartonella-containing blood produce anemia in such treated animals, with a multiplication of the bodies on the red corpuscles. Blood without the bartonella fails to produce the anemia. On the basis of these transfers Mayer concluded that *Bartonella muris ratti* is a living organism and the etiological agent in rat anemia after splenectomy.

The presence of *Bartonella muris ratti* in the blood of anemic splenectomized rats has subsequently been confirmed by Lauda and Marcus (5) and similar findings have been reported by Bayon (6) in England, de Faria and Cruz (7) in South America and by Jaffé and Willis (8), Noguchi (9) and Cannon, Taliaferro and

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Dragstedt (10) in North America. Bodies resembling *Bartonella muris ratti* have been found in the blood of other species of normal animals and named *Grahamella*, but whether they are living organisms and have any relation to pathologic conditions has not yet been determined. *Bartonella muris ratti* has occasionally been observed in normal rats, notably by Lauda (5) and by Jaffé (8).

Bartonella-like organisms from rats have been cultivated for brief periods on artificial media by several observers, notably Mayer (11), Bayon (6) and Noguchi (9), but none of these cultivated organisms have been identified as the cause of the anemia owing to the lack of susceptible animals upon which to test them.

As a result of these investigations it may be regarded as established that in many strains of tame and wild rats splenectomy is followed by anemia and that this anemic blood always shows *Bartonella muris ratti* on the red cells. Thus far the anemia has not been reproduced in normal animals (*i.e. those still possessing the spleen*) nor has *Bartonella muris* been transferred successfully to normal animals.

During the past 2 years we have splenectomized a series of 66 rats with the following problems in mind:

1. To determine whether white rats in Baltimore develop anemia after splenectomy with *Bartonella muris ratti* on the red blood corpuscles.

2. To find some normal animal in which anemia can be produced by transfer of material (blood or organs) from anemic splenectomized rats.

3. To cultivate *Bartonella muris ratti* on artificial media.

4. To determine the relationship of *Bartonella muris ratti* to anemia.

A. The Anemia of Splenectomized Rats.

In two strains of rats available for this work, removal of the spleen is followed by an anemia like that described by Lauda and *Bartonella muris ratti* appears in the blood.

One is a strain of white rats purchased from a Baltimore dealer, the other a strain of hooded rats raised in the School of Hygiene. In a series of 58 rats some grade of anemia has appeared in all instances. In some cases the red cell count drops to between 2 and 3 million cells per c. mm. from an original count of 10–11 millions. The hemoglobin drops to 20–30 per cent (estimated by the Sahli hemoglobinometer) and hemoglobin appears in the urine. The white blood corpuscles rise markedly, the count increasing from an original count of about 16,000 to perhaps 35,000 per c. mm., and sometimes to 65 or 70,000. The increase is

largest in the polymorphonuclear neutrophils but the mononuclears also show a relative and absolute leucocytosis. At the same time there is apparently an increase of blood platelets, anisocytosis is often marked, there is polychromatophilia and a shower of normoblasts follows the destruction of red cells. The phagocytosis of red blood cells by the circulating mononuclears is characteristic. There may be as many as a dozen red cells in a single mononuclear. In all instances the blood of these anemic splenectomized rats contains *Bartonella muris ratti*. These structures appear on the 2nd to the 3rd day after splenectomy and their number corresponds to the grade of the anemia which subsequently develops.

Of the 58 rats splenectomized in the first series all developed bartonella anemia and 12 died of anemia from 7–12 days following the operation. 17 others died following bleeding from the heart for transfer of the virus to other animals. All of these showed a rapidly falling hemoglobin and many bartonellas on the red cells, and gave every promise of developing a severe and perhaps fatal anemia. 30 rats recovered from the anemia completely and showed a normal blood picture within about 4 weeks. 8 of these underwent a spontaneous or induced relapse 4–8 weeks later and in every case died of the anemia.

In addition 8 Littlestown rats were splenectomized which did not develop anemia nor show bartonellas except after exposure to infected rats.

The following protocol shows the blood changes in a splenectomized hooded rat which succumbed to the anemia.

Protocol for Rat 21.

Hooded Rat (*School of Hygiene Strain*).

Date	Hemo-globin	Red cells	White cells	Observations and operations
Jan. 7	93	10,730,000	15,000	
" 9	91	10,770,000		Splenectomized
" 10	80	8,780,000	15,800	
" 11	82	9,760,000	17,100	Occasional rods
" 12	80	9,870,000	15,400	Few rods
" 13	75	8,740,000	20,900	Many "
" 14	43	5,040,000	30,200	" "
" 15	21	2,160,000	19,700	" " polychromatophilia, anisocytosis
" 16	22	2,220,000	14,700	Fewer " " "
" 17	27	2,110,000	7,450	" "
" 18	27	2,690,000	6,950	" "
" 19				Found dead

Text-fig. 1 shows the characteristic blood changes in a splenectomized rat which developed a severe anemia, recovered and subsequently had a relapse.

B. *Production of Anemia in Normal Animals.*

Transfer to Young Rats.

Intraperitoneal or intravenous transfer of blood from anemic splenectomized rats has been without effect on normal adult rats (with certain exceptions to be noted later), normal adult guinea pigs,
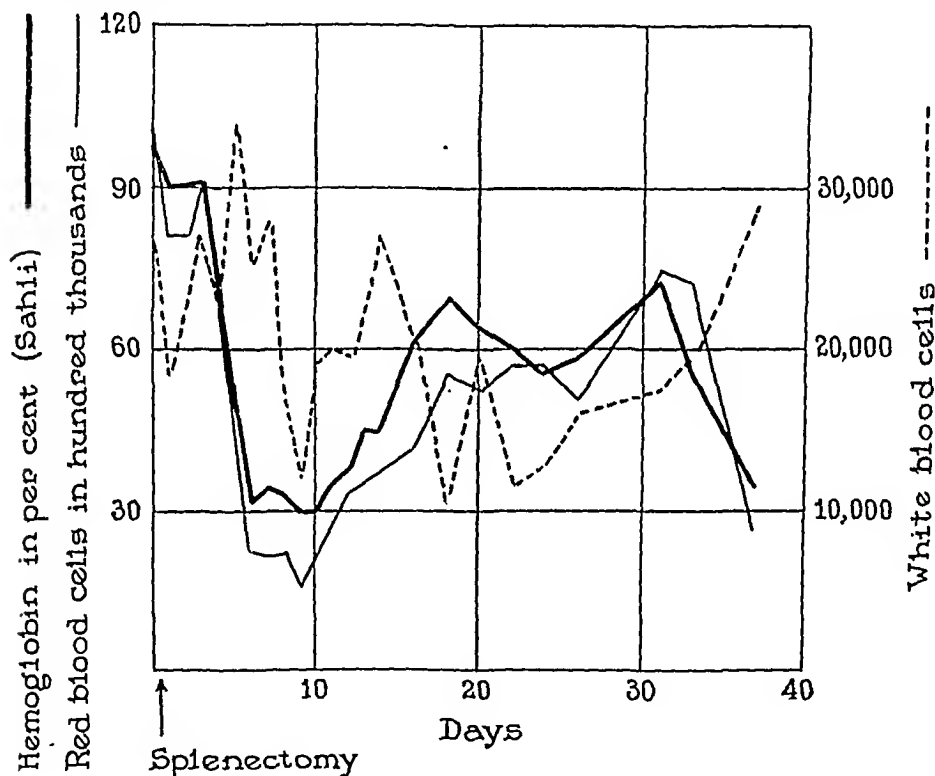
TEXT-FIG. 1.   Rat 22.   Splenectomized January 9.

normal adult rabbits and half grown dogs.   Anemia did not develop after the transfers nor did the blood show *Bartonella muris*.   Intraperitoneal injection of young rats about 3 weeks old, however, gave very different results.   The blood of anemic splenectomized rats, taken 3–6 days after removal of the spleen, will produce anemia in young normal rats with intact spleen, provided that these rats are not too old or too large.

Rats 20–30 gm. in weight give the best takes, rats 40 gm. in weight are usually less satisfactory and with 60 gm. rats inoculation is frequently without effect. The disease develops in these young normal animals in much the same way that it does in adult rats after splenectomy. *Bartonella muris* appears in the blood in 1–3 days and the blood count begins to drop. On the 4th or 5th day the count may reach a minimum of about 3,000,000 cells per c. mm. The hemoglobin drops correspondingly, often to 30–40 per cent from 70–80 per cent and hemoblogin may appear in the urine. The leucocyte count does not change. In normal young rats this count is usually about 8,000 per c. mm. and the count is not appreciably increased at any time after inoculation with anemic blood, nor is there any definite increase of blood platelets. This failure of increase of leucocytes and of blood platelets is the only difference which has been noted between the blood picture in the adult anemic splenectomized rats and the young rats which develop anemia after transfer of bartonella-containing blood. In severe cases the animals may die in 5–8 days and at autopsy the mucous membranes and the organs are very pale. There is some icterus of the skin, the liver looks fatty and hemoglobin may be present in the urine in the bladder. The spleen is often notably increased in size.

The following protocols show the effect of the inoculation of normal animals with blood from anemic splenectomized animals.

Protocol of Rat N143.

Young normal rat weighing 35 gm. Injected April 17 intraperitoneally with 0.3 cc. whole blood from Rat N142 (third transfer from original splenectomized Rat 57).

Date	Hemo-globin	Red cells	White cells	Operation and observations
Apr. 17	70	7,780,000	8,500	Injected 0.3 cc. blood
" 18	62	6,490,000	8,600	Occasional rod
" 19	54	5,525,000	8,370	Few rods
" 20	52	4,160,000	9,300	Rods, polychromatophilia, anisocytosis
" 21	44	3,850,000		Occasional rod polychromatophilia, aniso-cytosis
" 24	64	5,720,000	8,250	Occasional rod

Protocol of Rat III 14–1.

Young rat, 35 gm., injected intraperitoneally with 0.5 cc. blood from splenectomized Rat 23.

Date	Weight	Hemoglobin	Red cells	White cells	Operations and observations
	gm.				
Jan. 16	35.0	75	7,000,000		Injection 0.5 cc. blood
" 17					Many rods
" 18	37.0	48	3,230,000		" "
" 19		32	2,620,000		Occasional rod
" 20	39.0	28	2,160,000		Rods
" 21		26	2,300,000	3,300	Few rods. Died few hours later

Autopsy.—Liver yellowish, spleen mottled yellow and red. Bladder filled with blood-tinged urine, slight icterus of skin. Cells in liver and spleen show phagocytosed red blood cells.

Success in the transfer of the virus of rat anemia to normal animals depends on a number of factors. The best results have been obtained with well nourished rats on a good balanced diet which reach a weight of 30–40 gm. in about 3 weeks. Ill nourished rats which are older at 30–40 gm. weight are not so satisfactory as younger rats. Rats over 60 gm. are about as resistant as normal adult rats, at times however, a few bartonellas may appear and a light anemia develop. Moreover, the virus in the original splenectomized rats varies greatly in its potency. In some instances it seems to have little effect on the normal animals while in other cases it may give excellent takes. Blood in the early stages of the anemia, 2–4 days after splenectomy, contains more potent virus than after the peak of the anemia is reached, and during or after recovery when bartonellas have disappeared from the circulation, the blood in our experience invariably loses its ability to produce anemia.

Blood from these normal animals in which anemia has thus been produced contains the virus of the disease. On transfer to other animals anemia develops and *Bartonella muris* appears in the blood. Blood for transfer gives the best takes when the red blood cells show the heaviest infection with bartonellas. This is usually from 2–5 days after injection. This passage of the virus can be kept up for succes-

sive generations of normal animals. Nine different strains of the rat anemia virus have been obtained from time to time and carried through at least three generations. Of these one was carried through five generations, one through six generations, two through seven generations, one through nine generations and one through thirty generations.

After several passages the virus tends to become less potent so that a more moderate anemia develops, usually a hemoglobin reading of 40–50 per cent being the lowest. The bartonellas are less numerous in the blood, that is, fewer cells are infected and there are fewer bodies in each infected cell. These are usually the large deeply staining pleomorphic forms that appear after active multiplication has ceased, and which seem to be a resting or resistant phase of the bartonella. The virus which we have carried through thirty generations went through several periods of this apparently inactive phase in which little or no anemia is produced. The bartonellas are carried along from transfer to transfer, undergo multiplication for a period of perhaps 24 hours and then recede promptly. By making several successive transfers at 24 hour intervals into younger and smaller rats we were able to bring this virus back into a state of active multiplication on two different occasions. Whether our more rapid transfer and choice of young rats were really the deciding factors in reviving the bartonella anemia is impossible to say. It is theoretically possible that the virus must go through some sexual or symplastic cycle outside of the blood stream at certain intervals, and so renew its vegetative energy. At all events its virulence and the morphology of *Bartonella muris ratti* seem to vary from time to time. The smallest injection with which we have produced a good take in a young rat has been 1/20 cc. of infected blood, but 1/100 cc. is sufficient to cause the appearance of bartonellas in the blood stream after 24 hours, and 1/200 cc. after 48 hours. These small injections, however, are apparently easily handled by the host. The bartonella is destroyed or its multiplication inhibited, and no anemia develops. Our usual infecting dose is 0.2–0.5 cc. injected intraperitoneally.

In all, 212 young rats have been injected with blood from other anemic rats. Of these, eight have died with a fatal anemia in 5–8 days, while fifteen others died, probably of anemia, but the terminal records were not complete enough to be absolutely convincing.

Forty-one died after bleeding from the heart for transfers.   As in the cases of the splenectomized rats, the best and most promising cases were always selected for transfer, so that many of these, killed in the early stages of the disease, might have gone on to a fatal issue, had they not been killed.   Thirty-nine of the young rats died from causes which could not be directly connected with the anemia.

Transfer to Young Rabbits.

In addition to young rats, young rabbits have also been found susceptible to the virus of rat anemia.   On three occasions young rabbits about 3 weeks old have developed characteristic anemia after intravenous inoculation of $\frac{1}{2}$ to 1 cc. of bartonella-containing rat blood.   The blood count dropped to about 3,000,000 from 6,000,000 and the hemoglobin to about 20 per cent (Sahli).   Bartonellas appeared on the blood corpuscles in 5–12 days and the height of the anemia was reached 3–7 days later.   In none of these animals did the infection proceed to a fatal issue.   The bartonellas rapidly disappeared from the blood and the blood count returned to normal.   There was a leucocytosis at the peak of the anemia.

Blood from these rabbits, containing *Bartonella muris*, again produced a characteristic bartonella anemia in young rats, showing that the virus can be passed through rabbits and not lose its potency for rats.

The following protocol shows the production of anemia in young rabbits.

Protocol Rabbit 17.

Young normal rabbit 3 weeks old. May 26, injected with 1 cc. blood intravenously from splenectomized Rat 17.

Date	Red cells	White cells	Operations and observations
May 26	5,672,000		Injected 1 cc. blood Rat 17
" 27			No rods
" 28			" "
" 30			" "
" 31	6,128,000	9,200	Occasional rod
June 1			" "
" 2			" "
" 3			" "
" 4			" "
" 7	4,616,000	9,600	Few rods, polychromatophilia
" 8	3,936,000	9,000	Many rods
" 9	3,448,000	7,600	" " anisocytosis
" 10	3,040,000	6,800	Fewer "
" 11			Few "
" 12	2,836,000	13,360	Occasional rod
" 13	4,712,000		
" 14	4,608,000	8,400	
" 18	5,280,000	21,400	

"Littlestown" Rats.

Another strain of rats purchased from a dealer in Pennsylvania and designated by us as the "Littlestown" strain, did not develop anemia nor show *Bartonella muris* after splenectomy. There are some rats, therefore, which do not harbor the virus of rat anemia and do not come down with the disease until subsequently infected either by artificial injection of virus or by some natural mode of transfer. This is similar to the experience of Jaffé and Cannon who find some strains of rats always infected and some never, or only occasionally so. We have thus far splenectomized five animals in this group, which when kept in isolation did not show *Bartonella muris* nor develop anemia even after several months. In three others, which had been kept in the animal house in the same room with infected rats for several

months, splenectomy was followed by anemia with the appearance of bartonellas in the blood. Such adult Littlestown rats which do not harbor the virus are susceptible to the virus upon injection. After inoculation of 1 cc. of blood from an anemic animal, a few bartonellas appear on the blood cells and a light anemia develops. This is never as severe as in the young rats, and rapidly regresses, the bartonellas disappearing and the blood count returning to normal.

Bartonella muris ratti in Wild Rats.

All of the white and hooded rats used for splenectomy or transfer have been examined for bartonellas before operation or injection and in only one instance have we seen the rods. This was a young rat of the Littlestown strain which had been in the laboratory 2 weeks. In all we have examined about 300 rats. Wild rats on the other hand have shown a high incidence of bartonellas on the circulating blood cells. Out of thirty-nine examined, ten showed bartonellas. The rods were never numerous, usually only one or two in about four oil immersion fields, in two cases several on one cell in nearly every field. The rat in this series showing the heaviest infection with bartonella had many trypanosomes in the blood and a heavy infection with leptospiras in the kidneys. This is in accord with Mayer's observation that rats infected with trypanosomes may show bartonellas.

C. Spontaneous Infection.

As mentioned above, in the two strains of School of Hygiene rats, removal of the spleen is always followed by the appearance of *Bartonella muris* and a more or less severe anemia. Another strain of white rats (Littlestown strain) obtained from a dealer in Pennsylvania and reared in a room remote from infected rats showed no bartonellas and did not develop anemia following splenectomy. Others of these rats splenectomized about a month after removal to the common animal room however, came down with anemia in the usual way. If the uninfected splenectomized rats now are put in a cage with infected splenectomized rats they begin to show bartonellas in the blood in 7–14 days and usually come down with a fatal anemia

a few days later.   Similarly, convalescent splenectomized rats under-
go relapses if placed with infected rats.

These observations indicate the highly contagious nature of the
virus of rat anemia and point rather strongly to an insect vector.
Cages and food cups are sterilized with steam twice weekly and care
is taken not to mix food or water cups from cage to cage.   The
chances for transfer of the virus by direct contact with feces of in-
fected animals or by biting are therefore remote.   All of our rats are
infested with *Polyplax spinulosa*.   An occasional adult *Cimex lec-
tularius* has been found around the cages and young forms have been
picked from some of the rats.   Experiments are under way to deter-
mine if these lice and bedbugs can transmit the virus to uninfected
animals.

D. Attempts to Cultivate Bartonella muris ratti.

Repeated attempts have been made to cultivate *Bartonella muris
ratti* on artificial media.   For this purpose all the usual laboratory
media have been utilized and in addition Noguchi's tissue media and
leptospira media.   Rat infusion agar from young normal animals
was prepared and the reaction adjusted to pH 6.4, 7.0 and 7.4.   Blood
of rabbits, horses and rats has been used for enrichment.   Thus far
no successful cultivation has been effected and it seems very doubtful
to us whether the organism has ever been grown artificially.   Tissue
cultures of blood or organs from infected animals have yielded no
conclusive results.

E. Viability of the Virus.

Blood drawn from the heart of an infected rat into a glass syringe
with heparin or sodium citrate remains infective for young rats for
at least 2 hours.   After 24 hours in the ice box the virus is definitely
attenuated, ordinary doses giving only a light take with few bartonel-
las in the blood and little or no anemia.   24 hours at room temperature
or 37°C. completely destroys the virus.   The virus and the bartonellas
are killed by heating the blood at 57°C. for $\frac{1}{2}$ hour.   Injection of
this heated blood causes neither anemia nor the appearance of bar-
tonellas.

Contrasted with the short life of the virus *in vitro*, is its apparent indefinite survival in the animal body. A young rat injected with bartonella blood and kept in isolation for several months following recovery from anemia, will upon splenectomy again come down with typical bartonella anemia. A rat from the uninfected strain kept in similar isolation shows neither bartonellas nor anemia following splenectomy. Rats from the non-infected strain injected with bartonella blood or merely exposed for a period of 2 weeks or so to infection, develop bartonella anemia upon subsequent splenectomy. Rats therefore become bartonella carriers, either after exposure or as a result of injection and apparently remain carriers indefinitely. Any rat harboring bartonellas will develop anemia following splenectomy even though it may have had and recovered from a typical case by injection.

F. Immunity.

These cases of anemia in splenectomized rats which have once had the disease, and the spontaneous and induced relapses in recovered splenectomized rats point to the failure of the immune mechanism in the absence of the spleen. Moreover, serum from a normal adult animal will not protect a splenectomized animal from invasion by bartonella and the development of anemia, nor will it protect the young injected animal from the disease.

Natural infection among the rats presumably is effected by minute doses, either through the bite of an insect or the ingestion of infected material. Such doses are easily handled by the immune mechanism, *Bartonella muris* does not multiply on the blood cells and no anemia develops. This accounts for the failure to find demonstrable bartonellas in normal white rats, although there may be occasional organisms present in the circulating blood at times. The relatively enormous doses of virus given young rats in our injections so overwhelm the immune mechanism that infection readily occurs and anemia develops. The younger the rat the more severe the disease, and the more likely to be fatal. Normal adult rats cannot be infected even with large doses. Removal of the spleen however is all that is necessary to precipitate the latent infection. Why the young spleen is

unable to protect the animal against invasion with this virus and the adult spleen is usually so completely effective, is a matter for future investigation. As mentioned before, however, the adult "Littlestown" rat which is normally not infected, can be made to undergo a light attack after injection which indicates that previous infection probably has some influence on the protective power of the spleen. At all events, it appears evident that the immunity developed in growing rats is primarily dependent on the spleen.

With this in mind it was considered important to see if the adult splenic tissue acted as a reservoir of inhibitory substances for the bartonella anemia. The spleen from a normal rat was ground in a mortar with a little physiological saline solution and allowed to stand for 1 hour with half the quantity of blood from an anemic splenectomized animal. $\frac{1}{2}$ cc. of the mixture was then injected into a young rat and a control rat was injected with an equal amount of blood without splenic tissue. The experiment was repeated four times, and in no case could we find any demonstrable difference between the control animals and the rats with splenic tissue. Both developed bartonella and a light or moderate anemia according to the potency of the virus used.

Attempts to produce an immune serum in rabbits have so far proven unsuccessful. A rabbit which has once had bartonella anemia cannot be made to undergo a relapse by further injections. Serum from such an "immune" rabbit which has recovered from the anemia and been given two subsequent injections of bartonella blood fails to protect splenectomized rats from infection and anemia. This is similar to Noguchi's experience with *Bartonella bacilliformis*.

G. *Nature of Bartonella muris ratti.*

In view of our inability to cultivate *Bartonella muris*, some question may arise as to its character, whether it is really a bacterium or represents a stage in the life cycle of some protozoan parasite.

According to our observations *Bartonella muris ratti* is a small bacillary body about 0.1 μ in width and 0.5–1.0 μ in length, which appears to lie on the surface of the red cells (Fig. 1). Stained by Wright's blood stain it takes a bluish hue with

the suggestion of redder granules at the ends. Stained by Giemsa the whole rod has a redder hue. It is decolorized by Gram's method and takes the fuchsin counterstain very faintly. When the rods first appear they are thick, dark and short, often diplococcoid, occur singly and only on an occasional cell. These forms are followed by dumb-bell-shaped or granular rods and long slender bacillary bodies. More cells are infected and there may be two or four rods on a cell. As the infection progresses the rods appear shorter, usually in parallel rows and chains and the majority of the red cells contain from a few to several dozen. Any time from the 5th–10th day after splenectomy, the majority of the infected cells disappear rather suddenly from the blood stream, and the remaining infected cells reveal larger deeply staining, highly pleomorphic bodies, with occasional peculiar prolongations of the cytoplasm. There may also be ring forms, thicker and more deeply stained on one side. These forms, which seem to be related to the earlier ones, suggest a stage in the life cycle of some protozoan.

Occasionally groups of bartonellas are found free in the plasma, but these are usually associated with fragments of laked corpuscles. The bartonellas do not stain well when they are free from the corpuscles, hence it is more difficult to identify them with certainty, particularly to distinguish them from granules of broken platelets. The platelet granules differ from the rods however, by their greater thickness and less delicate outline and the redder hue with Wright's stain. The rods are also readily distinguished from the basophilic granules of the red cells, from chromatin dust and the Howell-Jolly bodies, all of which tend to increase during the course of anemia. The reticulated red cells also increase during the infection. By staining with cresyl blue and then by Wright's the difference between the bartonellas and reticulum is well brought out. The reticulum appears as bluish rods or masses of rods principally in the center of the cell, while the bartonella rods are distinctly redder and appear toward the periphery of the cell. Only a few reticulated cells are infected with bartonellas and then usually very lightly. We have not been able to see *Bartonella muris* in unstained smears or on living cells in the hanging drop. Fresh dark field preparations, however, reveal the bartonellas on the cells as non-motile rods. They apparently do not stain with neutral red. Many cells in bartonella anemic blood may show neutral red-stained rods, but from their position and distribution we believe that they are reticulum rather than bartonellas.

H. Relation of Bartonella muris ratti to Rat Anemia.

Since *Bartonella muris* has never been grown artifically, and rat anemia produced by cultures, it cannot be regarded definitely as the cause of the disease. There is, however, some collateral evidence which makes it highly probable that this organism stands in etiological relation to it. *Bartonella muris* is always associated with the anemia

and varies with the extent of blood destruction.   If it appears on the corpuscles in large numbers or if many corpuscles harbor it the anemia is apt to be severe.   When only a small number appear in the blood, the anemia is mild.   Moreover, the bartonellas seem to be destroying the cells.   In heavily infected blood one can find all stages of blood destruction, frequently broken down corpuscles and shadows of cells with bartonellas still clinging to them.   Only blood containing *Bartonella muris* can produce the anemia.   Blood in which bartonellas cannot be demonstrated has regularly failed to produce it.   Red blood corpuscles harboring bartonellas, freed from serum and washed repeatedly in physiological saline, produce the anemia.   The bartonellas can be demonstrated in stained films of these washed cells.   The plasma removed from these same cells does not produce immediate anemia after injection.   That a few bartonellas are actually present, however, is demonstrated by their appearance in small numbers in the injected rat after a prolonged incubation period and the subsequent production of a moderate anemia.   Blood from rabbits in which a mild anemia has been caused by inoculation of bartonella rat blood and which harbors *Bartonella muris*, will produce a characteristic anemia in young rats which again show the bartonellas.   As pointed out above, the virus producing the anemia and the bartonellas are both destroyed at 57°C.   It can be said, therefore, that red blood corpuscles plus *Bartonella muris* produce anemia and that *Bartonella muris* either represents the virus or that the virus is attached to the corpuscles and *Bartonella muris* is an accompanying organism.

The following experiment illustrates the production of bartonella anemia in young rats by the injection of whole blood or washed cells and the failure of plasma to cause anemia except after a prolonged incubation period.

Experiment with Virus 30.

Rat 30 bled from heart 5 days after splenectomy. Rat N9 injected with 0.2 cc. and Rat N10 with 0.3 cc. whole blood. Blood centrifugalized. Rat N12 injected with 0.5 cc. plasma. Cells washed in physiological salt solution, resuspended and 0.5 cc. injected into Rat N13.

```
                    ┌─────────┐
                    │ Rat 30  │
                    └────┬────┘
        ┌───────────┬────┴──────┬───────────┐
   ┌────┴───┐  ┌────┴───┐  ┌────┴───┐  ┌────┴────┐
   │  R N9  │  │ R N10  │  │ R N12  │  │  R N13  │
   └────────┘  └────────┘  └────────┘  └─────────┘
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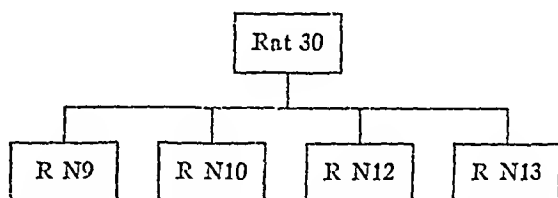
	R N9	R N10	R N12	R N13
Weight	30 gm.	32 gm.	24 gm.	32 gm.
Injection	0.2 cc. whole blood	0.3 cc. whole blood	0.5 cc. plasma	0.5 cc. washed cells
Bartonella				
2 days	Many	Many	None	Many
10 days	Occasional	Few	Few	Occasional
Hemoglobin				
Before injection	75	77	80	72
1 day after injection	67	65	82	70
2 days " "	48	53	78	70
3 " " "	30	41	80	24
4 " " "	31	36	78	27
10 " " "	65	48	50	65

SUMMARY AND CONCLUSIONS.

Fifty-eight white and hooded rats have been splenectomized and all of them have shown a more or less severe anemia and an infection of the red blood cells with *Bartonella muris*. Another strain of white rats obtained from Littlestown showed no anemia and no bartonellas in the blood after splenectomy, until exposed to infected rats. Others of these Littlestown rats, kept in the laboratory for some time before operation and exposed to infected rats, came down with bartonella anemia within 6 days after splenectomy.

Whole blood or the washed red blood corpuscles from splenectomized rats which show bartonellas and anemia will produce a similar condition in young rats when injected intraperitoneally. Adult rats of

strains which harbor the virus (as demonstrated by splenectomy) cannot be infected by injection.

Intravenous inoculation of young normal rabbits with blood from an infected rat will sometimes produce a similar infection and anemia in the rabbit, and the virus can then be transferred back to young rats.

The virus of rat anemia may be transferred from young normal rat to young normal rat with the appearance of *Bartonella muris* and the production of anemia. In the early transfers the disease may be fatal, but it usually becomes milder in successive passages.

Although we have not yet been able to cultivate *Bartonella muris* and prove its etiological relationship to rat anemia by inoculation of cultures, we have added to the evidence that *Bartonella muris* is the cause of the anemia. Washed red blood corpuscles, containing bartonellas, will produce the anemia in the usual way while plasma from the same cells will either fail to produce it altogether or only after a prolonged incubation period. Blood heated to 57°C. for $\frac{1}{2}$ hour fails to produce anemia or the appearance of bartonellas in the blood of inoculated animals.

From these observations the following conclusions may be drawn:

1. All rats which harbor *Bartonella muris ratti* come down with a more or less severe anemia after splenectomy.

2. Young rats which have not yet developed an immunity undergo the typical anemia after intraperitoneal injection of blood from a splenectomized animal in the early stages of the anemia.

3. Young rabbits may show bartonellas and develop anemia following intravenous inoculation of infected blood.

4. The virus of rat anemia and *Bartonella muris ratti* may be transferred from normal animal to normal animal for successive generations. Such strains have now been transferred for five, nine and thirty generations.

5. The resistance of rats to bartonella anemia is almost wholly dependent on the spleen. Other organs do not take over this function of protection as shown by the relapse of splenectomized rats many months after recovery. Young rats which have recovered from an attack of anemia are not protected by this previous infection from the invasion of the virus following splenectomy. Adult splenic tissue

mixed with infected blood before injection does not inhibit or neutralize the virus.

6. The virus of rat anemia is highly contagious and rats exposed to infection acquire it in some unknown way.

7. *Bartonella muris ratti* represents the virus of rat anemia or at least cannot be separated from the virus because:

(*a*) The anemia in splenectomized and injected animals is always preceded by the appearance of bartonellas and the grade of anemia is proportional to the degree of infection with bartonellas.

(*b*) Washed corpuscles containing bartonellas always produce anemia. Plasma either fails to do so, or produces a mild anemia after a long incubation period with a few bartonellas in the blood.

(*c*) The thermal death point of virus and bartonella is the same.

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EXPLANATION OF PLATE 11.

Fig. 1. *Bartonella muris ratti* in the blood from a rat with severe anemia, 5 days after splenectomy. Stained by Wright's stain.
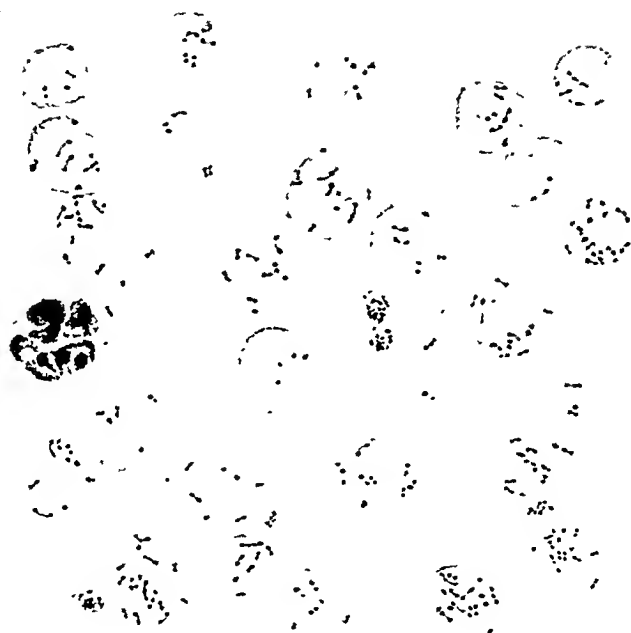
FIG. 1.

THE CONVERSION OF HEMOLYTIC STREPTOCOCCI TO NON-HEMOLYTIC FORMS.

By E. W. TODD,* M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

Bacterial variation has been studied by a number of workers and it is now a well established observation that avirulent variant cultures can be obtained from a number of microorganisms. Virulent cultures usually form smooth colonies on agar and avirulent variants are distinguishable by the rough appearance of their colonies. In the case of hemolytic streptococci, however, it has been shown (1) that these relations are reversed, that smooth colonies characterize an avirulent culture and that the rough form of colony indicates that a culture is either actually or potentially virulent. To avoid confusion, which might arise from this unusual relationship between virulence and colony form, the virulent type of colony has been designated the "matt" form and the avirulent type has been called the "glossy" form. A matt culture may be either virulent or attenuated but its virulence can always be enhanced by animal passage.

A number of matt strains of hemolytic streptococci were subjected to mouse passage; and in the course of these experiments two strains were encountered, both derived from cases of puerperal septicemia, which became progressively less hemolytic on blood agar as they were passed through a series of mice. One of these strains retained some hemolytic power after thirty mouse passages but the other strain (Henson) was completely deprived of its hemolytic properties by passage through five mice. At first the non-hemolytic passage culture was rejected on the theory that it arose from contamination in the peritoneal cavity but it was subsequently found that this strain could always be rendered non-hemolytic by passage through a few mice. Young colonies of the passage culture,

which were opaque, greenish in color and non-hemolytic, did not affect the blood cells in the agar surrounding the colonies; but older colonies were surrounded by a narrow green zone. This non-hemolytic culture was passed through 74 mice and the peritoneal washings and hearts' blood of the mice were plated out daily on blood agar but the colonies remained completely non-hemolytic throughout the experiment. In contrast to this result the hemolytic character of the glossy variant of the same strain remained unaltered after passage through thirty mice.

The non-hemolytic culture was reverted to the hemolytic form by continued daily subcultivation on artificial media. Twenty transfers in broth caused the reappearance of a distinct halo of hemolysis around each colony but the degree of hemolysis remained subnormal until the culture had been transferred in broth 150 times. After this large number of transfers the culture was partially converted to the glossy state and two forms of colonies now appeared on blood plates, the matt form which was moderately hemolytic and the much more hemolytic glossy form. Broth cultures of the two forms were obtained by selecting suitable colonies and the passage of these two cultures through mice confirmed the previous experiments—the matt form became non-hemolytic but the glossy form retained its hemolytic properties.

It will be seen from the above description that three forms of the same strain, showing different degrees of hemolysis, were available:

1. The original matt culture was moderately hemolytic on blood agar and was avirulent for mice. (M.L.D. 0.5 cc.)

2. The glossy avirulent form, derived from the original culture by long continued subcultivation on artificial media, was much more hemolytic than the original form and its hemolytic properties were unaltered by mouse passage. (M.L.D. 0.5 cc.)

3. The non-hemolytic form, obtained from the original culture by mouse passage, was moderately virulent. (M.L.D. 0.00001 cc. after 47 passages.) Long continued subcultivation of this form caused it to revert to a mixture of the other two forms.

The Effect of Reduced Oxygen Tension on the Hemolytic Properties of Three Forms of Streptococcus hæmolyticus.

The effect of reduced oxygen tension on the three forms of culture was investigated. Colonies of the passage culture were completely non-hemolytic when freely exposed to oxygen on the surface of blood agar plates; but hemolysis became evident when the supply of oxygen was diminished by growing the culture in blood broth or in the depths of blood agar. When oxygen was more completely excluded, by cultivation in an anaerobic jar, the passage culture, and the original culture, both formed hemolytic colonies on the surface of blood agar

TABLE I.

Showing the Comparative Amount of Hemolysis, Which Surrounded Colonies of the Three Forms of Culture, under Different Degrees of Oxygenation.

	Surface colonies on blood agar plates in air	Surface colonies on anaerobic blood agar plates	Deep colonies in blood agar (aerobic incubation)
Original culture.............................	++	++	++
Passage culture.............................	0	+	+
Glossy variant.............................	+++	+++	+++

0 = no hemolysis.

+++, ++, + represent the various degrees of hemolysis.

and the zones of hemolysis surrounding colonies of the two cultures were almost equal in circumference.

Table I gives the degree of hemolysis of the three forms of the same strain, on the surface of blood agar plates in air, on the surface of anaerobic plates and in the depths of blood agar.

Reed, Orr and Campbell (2) have shown that the filtrate from a culture of *B. welchii* has greater hemolytic activity on a suspension of reduced blood cells than on a similar suspension of oxygenated cells. The following experiment was done to determine whether the hemolytic activity of the passage culture of Strain Henson, which only appeared under anaerobic conditions, was due to the effect of diminished oxygen tension on bacterial metabolism or to the reduced state of the blood cells.

A 5 per cent suspension of rabbits' washed red blood cells in salt solution was divided into two parts, both of which were sealed with paraffin oil; one portion was reduced by passing hydrogen through the suspension for an hour and the other part was oxygenated by similar treatment with air. Hemolysin was prepared by filtering a culture of the original form of Strain Henson after 10 hours incubation; progressive dilutions of the filtrate in infusion broth were divided into two parts so that two sets of serial dilutions (volume 0.5 cc.) were obtained and, after all the tubes had been sealed with paraffin oil, 0.5 cc. volumes of reduced cells were

TABLE II.

Showing the Hemolytic Activity of a Filtrate from the Original Form of Strain Henson on Reduced and on Oxygenated Blood Cells.

Dilution of filtrates	Hemolytic activity of the filtrate on	
	Oxygenated cells	Reduced cells
1 in 2	++++	++++
1 in 4	++++	++++
1 in 8	++++	++++
1 in 16	+++	+++
1 in 32	+++	+++
1 in 64	+++	+++
1 in 128	++	++
1 in 256	+	+
1 in 512	+	+
1 in 1,024	—	—
Control	—	—

In all tables the following symbols are employed:

++++ = complete hemolysis.
+++ = almost complete hemolysis.
++ = incomplete hemolysis.
+ = slight hemolysis.
— = no hemolysis.

added to one set of dilutions with a capillary pipette and the same amounts of oxygenated cells were added to the other series. Readings were taken after the test had been incubated for 1 hour and the results, which are recorded in Table II, showed that the hemolysin was equally active on reduced cells and on oxygenated cells.

No steps were taken to exclude oxygen from the dilutions before the addition of the cells but, as the reduced cells retained their characteristic cherry-red color throughout the test, the conditions were considered satisfactory.

McLeod and Gordon (3) showed that peroxide is formed in broth cultures of pneumococci and Avery and Morgan (4) demonstrated that the quantity of peroxide formed depends on the surface area of broth exposed to the air.   They found that strongly positive peroxide reactions could be obtained when a broth culture was incubated in a shallow layer allowing free access of oxygen, that deep broth cultures, with only a small surface area, produced smaller amounts of peroxide and that anaerobic cultures did not form any peroxide.   They also
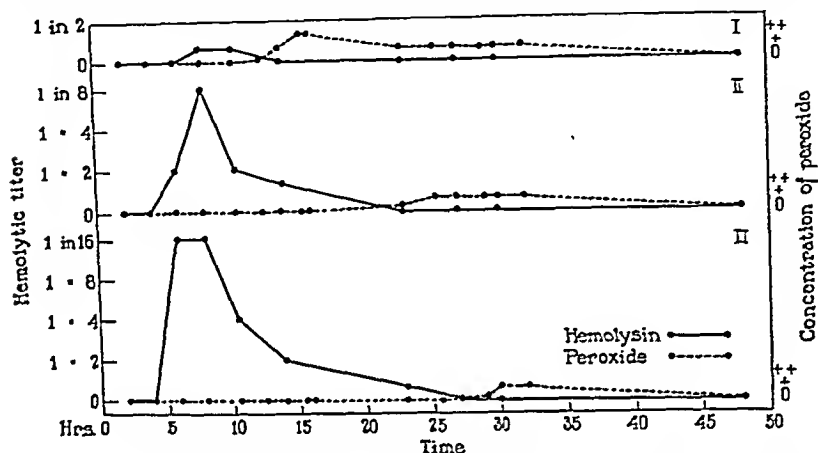
CHART 1.   Showing the times at which hemolysin and peroxide appeared in three different cultures of the same strain of hemolytic streptococcus.   I. Passage culture (non-hemolytic on aerobic blood plates).   II. Matt attenuated culture (moderately hemolytic on aerobic blood plates).   III. Glossy culture (very hemolytic on aerobic blood plates).

examined twenty-three strains of hemolytic streptococci and found that fifteen of these strains produced peroxide when grown in shallow layers of broth.   The observations of Avery and Morgan run parallel to those already recorded in this paper—free exposure to air, a condition which is necessary for the accumulation of peroxide, prevented the formation of hemolysin by our passage strain of hemolytic streptococcus; conversely anaerobic conditions, which prevent the formation of peroxide, were necessary for the production of hemolysin.

The following experiment was done to see if any connection could be established between the formation of hemolysin and peroxide in shallow broth cultures of hemolytic streptococci freely exposed to air.

Three 2 liter Erlenmeyer flasks, each containing 50 cc. of infusion broth to which 20 per cent of normal horse serum had been added, were sown with 0.2 cc. of young cultures of the three forms of hemolytic streptococcus (Strain Henson) and placed in the incubator together. Samples of culture were removed from the three flasks at intervals and tested for peroxide by the potato-benzidine reaction; periodical titrations of hemolysin were also made by centrifuging samples of the cultures and testing the supernatants against a 5 per cent suspension of washed rabbit cells. The results of this experiment are given in Chart 1.

The original culture of Strain Henson (II on chart) formed a moderate amount of hemolysin which first appeared after 6 hours incubation and disappeared after 23 hours; the disappearance of hemolysin was immediately followed by the appearance of peroxide for the first time.

The passage culture of Strain Henson (I on chart), which was non-hemolytic, when freely exposed to oxygen on the surface of blood agar, produced less hemolysin in broth than either of the other cultures; and a comparison of the three curves brings out two points which demonstrate this relative weakness in hemolytic power, (1) the low hemolytic titer, and (2) the fact that hemolysin appeared later and disappeared earlier from this culture than from either of the other cultures. This deficiency in hemolysin production was associated with the ability to produce more peroxide than either of the other cultures; but it is interesting to note that peroxide and hemolysin did not appear in the culture at the same time and that the disappearance of hemolysin was immediately followed by the rapid accumulation of peroxide.

The supernatant fluid from a centrifuged culture of the glossy variant (III on chart) gave the highest titer and longest duration of hemolysin; and peroxide, which was absent until the hemolysin had vanished, did not appear until the culture had been incubated for 29 hours.

Each of the three curves shows that peroxide and hemolysin did not occur simultaneously in the same culture.

Avery and Neill (5) have shown that hemotoxin is inactivated in cultures of pneumococci by the oxidizing action of peroxide; and their observations, coupled with the result of the experiment described above and also with the observation that the passage culture of Strain Henson was non-hemolytic in air but hemolytic under anaerobic conditions, suggested that the disappearance of hemolysin from cultures of hemolytic streptococci was also due to oxidation.

Is the Rate of Disappearance of Hemolysin Increased by Oxygenation of the Medium?

The following experiments show that the disappearance of hemolysin from cultures of hemolytic streptococcus is not influenced by oxygenation.

Experiment A.—Two cultures of the original form of Strain Henson were grown in 10 cc. of infusion broth containing 20 per cent normal horse serum—in one case the culture was made in a test-tube so that only a small area of broth was in contact with the air; in the other case the same volume of broth was placed in a 250 cc. Erlenmeyer flask forming a very shallow layer with a large area exposed to the atmosphere. Hemolysin was titrated after 6 hours incubation and again

TABLE III.

Showing that the Hemolytic Titer of a Culture of Streptococcus hæmolyticus is not Influenced by the Surface Area of Broth Exposed to the Air.

Dilution of supernatant	Hemolysin after 6 hrs. incubation		Hemolysin after 24 hrs. incubation	
	Shallow culture with large aerating surface area	Deep culture with small aerating surface area	Shallow culture with large aerating surface area	Deep culture with small aerating surface area
1 in 2	++++	++++	—	—
1 in 4	++++	++++	—	—
1 in 8	++++	++++	—	—
1 in 16	++	++	—	—
1 in 32	+	+	—	—

after 24 hours incubation. Table III shows that the different degrees of aeration in these two cultures did not cause any difference either in the titer of hemolysin or in the rate of its disappearance.

Experiment B.—Two cultures of the glossy form of Strain Henson were grown in shallow layers of broth in 250 cc. Erlenmeyer flasks; one flask was incubated in the air and the other in an anaerobic jar. Samples from the aerobic culture were tested for hemolysin at frequent intervals and as soon as the tests became negative the anaerobic jar was opened and the second culture was found to be free from hemolysin.

Experiment C.—A hemolytic filtrate, obtained from the glossy form of Strain Henson, was divided into two parts; one part was incubated anaerobically for 24 hours and the other part was incubated for the same time in air. Hemolysin had completely disappeared from both portions of the filtrate after 24 hours incubation.

The following experiments (D and E) were done to see if the addition of the reagent hydrogen peroxide to an actively hemolytic filtrate would reduce the hemolytic titer by oxidizing the hemolysin.

Experiment D.—Various quantities of hydrogen peroxide ranging from a dilution of 1 in 20 to a dilution of 1 in 2,000 were added to aliquot parts of a filtrate, prepared from a culture of the glossy variant of Strain Henson, and broth controls containing the same quantities of hydrogen peroxide were set up at the same time. After standing at room temperature for an hour the mixtures were tested for hemolysin and for peroxide.

TABLE IV.

Showing that the Hemolytic Titer of Supernatant Fluid from a Culture of Streptococcus hæmolyticus is Not Altered by the Addition of Hydrogen Peroxide.

Dilution of supernatant	Hemolytic titer of filtrate before addition of hydrogen peroxide	Hemolytic titer of filtrate 1 hr. after the addition of various quantities of hydrogen peroxide			
		Control. No H$_2$O$_2$ added to filtrate	H$_2$O$_2$ + filtrate 1 in 20	H$_2$O$_2$ + filtrate 1 in 200	H$_2$O$_2$ + filtrate 1 in 2,000
1 in 2	++++	++++	++++	++++	++++
1 in 4	++++	+++	+++	+++	+++
1 in 8	+++	++	++	++	++
1 in 16	+++	++	++	++	++
1 in 32	++	+	+	+	+
1 in 64	++	+	+	+	+
1 in 128	+	−	· −	−	−
Control hemolytic test with broth to which the same quantities of H$_2$O$_2$ had been added..................		−	−	−	−
Peroxide test 1 hr. after the addition of various quantities of H$_2$O$_2$..............		−	++++	+++	−

It will be seen from Table IV that hydrogen peroxide did not alter the titer of hemolysin.

Experiment E.—The last experiment was repeated with the following modifications: Two 25 cc. volumes of hemolytic filtrate from the glossy variant of Strain Henson were placed in flasks and the same amount of broth was placed in a third flask. Equal quantities of hydrogen peroxide, making a dilution of 1 in 100, were added to one flask of filtrate and to the control broth. The three flasks, contain-

ing respectively, (1) untreated filtrate, (2) the same filtrate with the addition of hydrogen peroxide and (3) broth similarly treated with hydrogen peroxide, were then incubated at 37°C.; and the hemolytic titer of each specimen was tested at intervals of 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours and 23 hours. Peroxide tests were frequently made during the course of this experiment and the supply of peroxide was replenished on the two occasions when these tests gave negative results.

Periodical titrations demonstrated a gradual fall in the hemolytic power of the two filtrates but the rate of disappearance of hemolysin was not affected by the addition of peroxide as the falling titers ran parallel up to the 23rd hour when hemolysin had disappeared from both specimens.

Experiment F.—In this experiment, which again shows that peroxide does not destroy the hemolysin of hemolytic streptococci, a peroxide of bacterial origin was used instead of the reagent hydrogen peroxide. A culture of a microorganism, resembling *Micrococcus tetragenus*, which was known to produce large quantities of peroxide, was incubated in infusion broth containing 20 per cent of normal horse serum for 30 hours; it was then filtered and the filtrate, which gave a strongly positive peroxide reaction, was divided into two parts. The peroxide was removed from one portion of filtrate by adding a small quantity of washed blood cells; and, after removal of the blood by centrifuging, the filtrate which now gave a negative peroxide reaction, was used to control the original filtrate.

A hemolytic filtrate, prepared in the usual way from the glossy variant of Strain Henson, was used to make the following mixtures:

    (1) 5 cc. hemolytic filtrate + 5 cc. broth.
    (2) 5 cc. hemolytic filtrate + 5 cc. tetrad filtrate (bacterial peroxide).
    (3) 5 cc. hemolytic filtrate + 5 cc. control tetrad filtrate (no peroxide).
    (4) 5 cc. tetrad filtrate (bacterial peroxide) + 5 cc. broth.

The hemolytic titer and the peroxide content of these mixtures were determined immediately and a second series of observations was made after they had been incubated at 37°C. for 1 hour.

Table V which gives the result of the experiment shows that the hemolytic titer remained unaffected by the bacterial peroxide.

The results of the experiments described in this section appear to indicate that the disappearance of hemolysin from cultures of hemolytic streptococci is not due to oxidation.

The Influence of pH on the Disappearance of Hemolysin from Cultures of Hemolytic Streptococci.

It is an old observation that hemolytic streptococci form non-hemolytic colonies on the surface of blood agar plates which contain glucose; and the following experiments were undertaken to determine the influence of pH on the rate of disappearance of hemolysin from cultures of hemolytic streptococci in broth.

TABLE V.

Showing that the Hemolysin of Streptococcus hæmolyticus is not Destroyed by Bacterial Peroxide.

Dilution of mixtures	Hemolytic titer immediately after mixing hemolysin and organic peroxide				Hemolytic titer of the mixtures after incubation for 1 hr. at 37°C.			
	Hemolysin + broth	Hemolysin + tetrad filtrate (bacterial peroxide)	Hemolysin + control tetrad filtrate (no peroxide)	Tetrad filtrate (bacterial peroxide) + broth	Hemolysin + broth	Hemolysin + tetrad filtrate (bacterial peroxide)	Hemolysin + control tetrad filtrate (no peroxide)	Tetrad filtrate (bacterial peroxide) + broth
1 in 2	++++	++++	++++	−	++++	++++	++++	−
1 in 4	++++	++++	++++	−	++++	++++	++++	−
1 in 8	++++	++++	++++	−	+++	+++	+++	−
1 in 16	+++	+++	+++	−	+	+	+	−
1 in 32	+	+	+	−	−	−	−	−
1 in 64	−	−	−	−	−	−	−	−
Peroxide tests....	−	++	−	++	−	−	−	−

Two flasks, containing respectively 20 cc. of plain infusion broth and 20 cc. of the same broth to which 1 per cent of glucose had been added, were sown with equal quantities of a young broth culture of the variant form of Strain Henson and were then placed in the incubator together. The hemolytic titer and the pH of samples from the two flasks were determined at intervals; and it will be seen from the two curves (I and II, Chart 2) that, in the culture which developed the larger quantity of acid owing to the fermentation of glucose, hemolysin appeared slowly, attained only a low titer and disappeared rapidly in comparison with the hemolysin of the less acid culture.

The next experiment shows that an acid reaction increases the rate at which hemolysin disappears from a filtrate.

A hemolytic filtrate (pH 7.4) was divided into two parts; the reaction of one part was adjusted to pH 8.0 by the addition of $\frac{N}{1}$ NaOH and the two portions were then placed in the incubator together. The hemolytic titers of the two portions of filtrate were determined before incubation, after $2\frac{1}{2}$ hours incubation at 37°C. and again after a further $17\frac{1}{2}$ hours at room temperature.

Table VI shows that the portion of filtrate which was adjusted to pH 8.0 retained its hemolysin longer than the untreated filtrate.
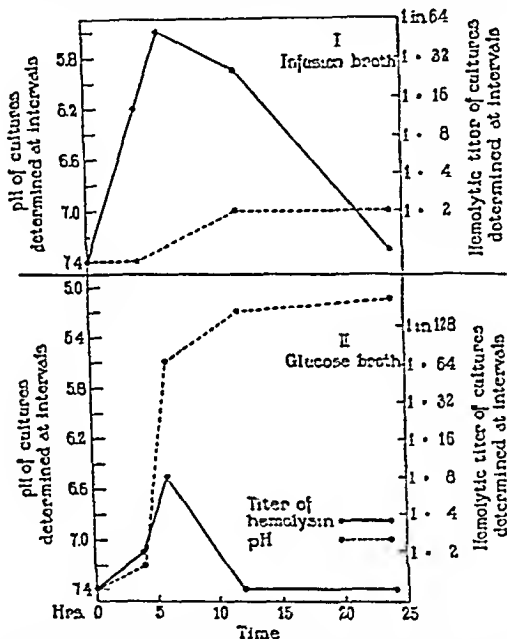
CHART 2. Showing the hemolytic titer and the pH of cultures of the same strain of hemolytic streptococcus. I. In infusion broth. II. In 1 per cent glucose broth.

Chart 3, which represents the result of a similar experiment, shows the rate of disappearance of hemolysin from two portions of a hemolytic filtrate which were kept in the ice box at 2°C. The portion of filtrate which was adjusted to pH 8.0 again retained its hemolysin longer than the untreated filtrate.

These experiments indicated that the rate of disappearance of hemolysin from cultures, and from filtrates, was dependent, to some

extent, on the reaction of the medium; and observations were therefore made to see whether the passage culture, which was non-hemolytic when exposed to air, produced more acid than the hemolytic glossy variant of the same strain. Shallow broth cultures of the two forms of Strain Henson which had been incubated long enough to produce the maximal quantity of hemolysin (6 to 8 hours) were filtered and the pH of the filtrates was determined: sometimes hemolytic filtrates from the glossy variant were more acid than hemolytic filtrates from the passage culture and, although slight differences of pH were usually found apparently due to the varying number of cocci which developed in the cultures, the reactions were always approximately equal and

TABLE VI.

Showing the Relation between the pH of a Filtrate and the Disappearance of Hemolysin.

Dilution of filtrate	Original hemolytic activity of filtrate		Hemolytic activity after 2½ hrs.		Hemolytic activity after 20 hrs.	
	pH = 7.4	pH = 8.0	pH = 7.4	pH = 8.0	pH = 7.4	pH = 8.0
1 in 2	++++	++++	++++	++++	+	+++
1 in 4	++++	++++	++++	++++	+	+++
1 in 8	++++	++++	+++	++++	+	++
1 in 16	++++	++++	+++	++++	−	++
1 in 32	++++	++++	++	+++	−	+
1 in 64	++++	++++	+	++	−	+
1 in 128	+++	+++	−	+	−	−

no constant preponderance of acid could be demonstrated in either culture.

The Cultivation of Different Forms of the Same Strain in Symbiosis.

The following experiments show the hemolytic titer of symbiotic cultures of the hemolytic and non-hemolytic forms of Strain Henson.

Three tubes of infusion broth, containing 20 per cent of normal horse serum, were sown in the following manner with young overnight cultures of the hemolytic and of the non-hemolytic forms of Strain Henson.

Tube 1 received 0.2 cc. of the non-hemolytic culture.
Tube 2 received 0.2 cc. of the hemolytic culture.

Tube 3 received 0.1 cc. of the non-hemolytic culture and 0.1 cc. of the hemo-
   lytic culture.

Ample oxygenation was assured by bubbling oxygen through the three cultures
during the whole period of incubation which was continued for 5½ hours.
The hemolytic titer of the three filtrates obtained from these cultures is shown in
Table VII.

The pure culture of the hemolytic form contained hemolysin but the other two
cultures contained no hemolysin.

The absence of hemolysin from the symbiotic culture might be
explained by at least two hypotheses: (1) that the non-hemolytic
form produced some substance in the presence of oxygen which not
only destroyed its own hemolysin but was also capable of destroying
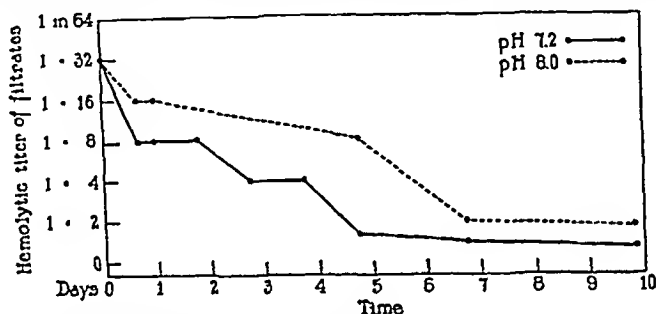
CHART 3. Showing the relation between the pH of a filtrate and the rate of
disappearance of hemolysin.

the hemolysin of the glossy variant; (2) that the non-hemolytic cocci
suppressed the multiplication of the hemolytic cocci.

To investigate the latter possibility various dilutions of the symbiotic culture
were plated out on the surface of blood agar and incubated in the presence of oxy-
gen; the number of hemolytic and of non-hemolytic colonies which developed on
the plates showed that the two forms had multiplied at approximately the same
rate, as 65 per cent of the 509 colonies counted were hemolytic. Differential
counts of hemolytic and non-hemolytic colonies were facilitated by the character-
istic translucence of the glossy hemolytic colonies and by the equally characteristic
opacity of the non-hemolytic matt colonies. Table VIII gives the result of a
similar experiment with the same strain of hemolytic streptococcus; but the
conditions were modified by omitting the oxygenation of the cultures and by
substituting cultivation in very shallow layers of broth. Three 2 liter Erlenmeyer
flasks, each containing 10 cc. of serum-infusion broth, were sown with the same

volumes of hemolytic and of non-hemolytic cultures as in the previous experiment; and after $5\frac{1}{2}$ hours incubation the cultures were centrifuged and the quantity of hemolysin in the supernatant fluids was determined.

It will be seen from Table VIII, first that the non-hemolytic culture formed some hemolysin in this experiment, possibly due to imperfect oxygenation, second that

TABLE VII.

Hemolysin in Filtrates Prepared from the Hemolytic and Non-Hemolytic Forms of Strain Henson and from a Symbiotic Culture of Both Forms.

Dilution of filtrates	Filtrates prepared from:		
	Pure culture of non-hemolytic form	Pure culture of hemolytic form	Symbiotic culture of both forms
1 in 2	—	++++	—
1 in 4	—	+++	—
1 in 8	—	++	—
1 in 16	—	+	—
1 in 32	—	—	—

TABLE VIII.

Hemolysin in Supernatant Fluid from Centrifuged Cultures of the Hemolytic and the Non-Hemolytic Forms of Strain Henson and from a Symbiotic Culture of Both Forms.

Dilution of supernatant fluid	Supernatant fluid from:		
	Pure culture of non-hemolytic form	Pure culture of hemolytic form	Symbiotic culture of both forms
1 in 2	++	++++	++++
1 in 4	+	++++	++
1 in 8	—	++++	+
1 in 16	—	++++	—
1 in 32	—	+++	—
1 in 64	—	++	—
1 in 128	—	+	—
1 in 256	—	—	—

the glossy variant was actively hemolytic, and, third that the symbiotic culture contained little more hemolysin than the pure culture of the non-hemolytic form. Differential counts showed that the symbiotic culture contained more than 50 per cent of hemolytic cocci.

The next experiment was done to determine the action of the non-hemolytic form of Strain Henson in symbiotic culture with an unrelated strain of hemolytic streptococcus (S43).

Two shallow layers of serum-infusion broth were sown with 0.2 cc. and 0.1 cc. of a culture of Strain S43 respectively; to the second was also added 0.1 cc. of a culture of the non-hemolytic form of Strain Henson. After 6 hours incubation the cultures were filtered and titrated for hemolysin content, as shown in Table IX.

Differential counts showed that the symbiotic culture contained 45 per cent of hemolytic cocci; a proportion which was not represented by the hemolytic titer

TABLE IX.

Hemolysin in Filtrate Prepared from an Unrelated Strain of Hemolytic Streptococcus (S43) and in Filtrate Prepared from the Same Strain in Symbiosis with the Non-Hemolytic Form of Strain Henson.

Dilution of filtrate	Pure culture of hemolytic streptococcus Strain S43	Symbiotic culture of hemolytic streptococcus Strain S43 and the non-hemolytic form of Strain Henson
1 in 2	++++	+++
1 in 4	++++	+++
1 in 8	++++	++
1 in 16	++++	+
1 in 32	+++	−
1 in 64	+++	−
1 in 128	+++	−
1 in 256	++	−
1 in 512	+	−
1 in 1,024	−	−

of the filtrate as the pure culture of Strain S43 contained considerably more than twice the quantity of hemolysin found in the symbiotic culture.

It was difficult to compute the quantity of hemolysin which might be expected to develop in a symbiotic culture if the non-hemolytic fraction of the mixed culture was inactive; and in order to gain some information on this point, and at the same time to reinforce the evidence already obtained that the destruction of hemolysin was not due to oxidation by peroxide, symbiotic cultures, composed of mixtures of *Streptococcus hæmolyticus* and *Streptococcus viridans*, were prepared. Five 250 cc. Erlenmeyer flasks each containing 10 cc. of serum-infusion broth were sown with the following quantities of culture:

Flask 1 received 0.2 cc. of the hemolytic glossy variant of Strain Henson.
Flask 2 received 0.2 cc. of the non-hemolytic form of Strain Henson.
Flask 3 received 0.2 cc. of *Streptococcus viridans* Strain A179.

Flask 4 received 0.1 cc. of the hemolytic glossy variant of Strain Henson and
　　0.1 cc. of the non-hemolytic form of Strain Henson.

Flask 5 received 0.1 cc of the hemolytic glossy variant of Strain Henson and
　　0.1 cc. of *Streptococcus viridans* Strain A179.

After $5\frac{1}{2}$ hours incubation the cultures were tested for peroxide and then filtered
immediately; Table X gives the quantities of hemolysin which the five filtrates
contained.　All the cultures gave negative peroxide tests with the single exception
of the pure culture of *Streptococcus viridans* which gave a faintly positive potato-
benzidine reaction.

The hemolytic form of Strain Henson produced, as usual, a good hemolytic
filtrate; the culture of *Streptococcus viridans*, and also the culture of the non-
hemolytic form of Strain Henson, produced no hemolysin.　The hemolytic titer

TABLE X.

*Hemolysin in Filtrates Prepared from Pure Cultures and from Symbiotic Cultures
of Strain Henson and Streptococcus viridans.*

| Dilution of filtrates | Hemolytic titer in filtrates prepared from: | | | | |
| | Pure cultures of: | | | Symbiotic cultures of hemolytic form of Strain Henson and | |
	Hemolytic form of Strain Henson	Non-hemolytic form of Strain Henson	*Streptococcus viridans*	Non-hemolytic form of Strain Henson	*Streptococcus viridans*
1 in 2	++++	—	—	+++	++++
1 in 4	++++	—	—	++	++++
1 in 8	++++	—	—	+	++++
1 in 16	++++	—	—	+	++++
1 in 32	++++	—	—	—	++++
1 in 64	+++	—	—	—	+++
1 in 128	+++	—	—	—	+++

of the symbiotic cultures showed that the hemolysin of the glossy variant was
partially destroyed in symbiosis with the non-hemolytic form of Strain Henson;
but symbiosis with *Streptococcus viridans* did not affect its output of hemolysin.

Differential counts on both the symbiotic cultures showed that the rate of
multiplication of the hemolytic cocci and of the non-hemolytic cocci was approxi-
mately the same in each case.

These experiments tend to show that the non-hemolytic form of
Strain Henson when cultivated in symbiosis with hemolytic cocci
not only destroys its own hemolysin in the presence of oxygen, but
also destroys the hemolysin of the associated hemolytic cocci.

Unfortunately more conclusive evidence of the destruction of hemolysin by the non-hemolytic form of Strain Henson was not obtained. Filtrates, prepared from the hemolytic form and from the non-hemolytic form, were mixed in equal proportions and a control was prepared by mixing together the same volumes of hemolytic filtrate and broth. Periodical titrations of these two mixtures showed that, when the pH was adjusted to the same level, hemolysin disappeared from the two mixtures at approximately the same speed.

Cultivation of the non-hemolytic forms in hemolytic filtrates did not give any further information, as the alteration of pH by the metabolism of the cocci was sufficient to cause hemolysin to disappear from the culture more rapidly than from the sterile control filtrate.

It is therefore uncertain whether the non-hemolytic form destroys the hemolysin formed by hemolytic streptococci or whether it produces conditions in the medium which prevent the formation of hemolysin.

DISCUSSION.

A number of workers have reported the mutation of *Streptococcus hæmolyticus* to *Streptococcus viridans* by animal passage. A similar change is recorded in this paper; but in this instance the loss of hemolytic activity was conditional on ample oxygenation and the organisms retained their hemolytic power in the absence of oxygen. It is probable that some of the previously reported "mutants" were also modified forms of hemolytic streptococci which, although they formed green colonies on blood agar, were possibly distinguishable from typical strains of *Streptococcus viridans* by their hemolytic activity in anaerobic culture.

The hemolytic streptococci, modified by animal passage, produced less methemoglobin on blood agar than typical green streptococci; and it would have been difficult to decide, on this criterion alone, whether they should be classified as green streptococci or as indifferent streptococci. The passage culture reverted to the hemolytic form after prolonged subcultivation on artificial media; and this reversion, coupled with the fact that the culture was always hemolytic in the absence of oxygen, served to identify the organism as a modified form of *Streptococcus hæmolyticus*.

The passage culture produced more peroxide than either of the

hemolytic forms of the same strain; and an attempt was therefore made to establish a causal relationship between the accumulation of peroxide and the disappearance of hemolysin. Periodical observations on broth cultures of the three forms of this strain showed that disappearance of hemolysin was concomitant with accumulation of peroxide; but attempts to demonstrate the destruction of hemolysin by peroxide were unsuccessful. It may be suggested that continued production of peroxide over a relatively long period, as by bacterial metabolism in a culture, is necessary for the destruction of hemolysin and that this condition was not established in the experiments recorded; but this explanation is untenable when we consider that anaerobic cultures of hemolytic streptococci, though unable to form peroxide, lost their hemolysin at the same rate as aerobic cultures which produced peroxide.

It is interesting to note that peroxide is preserved in an acid reaction while hemolysin is destroyed under the same condition; and it might be supposed that the pH of a culture determines the relationship between disappearance of hemolysin and appearance of peroxide, but this explanation seems inadequate because the different forms of hemolytic streptococci were approximately equal in their rate of acid production.

We are therefore forced to the conclusions that the hemolysin of *Streptococcus hæmolyticus*, in contrast to the apparently similar hemotoxins of pneumococci and other bacteria, is not destroyed by oxidation; and that the concomitant disappearance of hemolysin and appearance of peroxide have no causal relationship, though they may both be dependent on some unknown common cause.

The passage culture, though hemolytic in the absence of oxygen, establishes some unknown condition in the presence of oxygen which abolishes hemolysin and, when grown symbiotically with an ordinary strain of hemolytic streptococcus which is capable of forming hemolysin in the presence of oxygen, it reduces the hemolytic titer of the symbiotic culture either by destroying hemolysin or by preventing its formation.

SUMMARY.

From one strain of hemolytic streptococcus three forms were isolated, which produced three different degrees of hemolysis on the surface of blood agar in the presence of oxygen.

The original form was moderately hemolytic; the glossy variant was more hemolytic than the original form; and the third form, obtained by passing the original culture through mice, was non-hemolytic.

Under anaerobic conditions all three forms were hemolytic.

The non-hemolytic passage culture, in the presence of an ample supply of oxygen, not only destroyed its own hemolysin, which only appeared under anaerobic conditions, but was also able to destroy the hemolysin of other cultures of hemolytic streptococci.

It is possible that these observations may throw some light on experiments reported by a number of workers showing that *Streptococcus hæmolyticus* can be transmuted to *Streptococcus viridans* by animal passage.

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A STUDY OF THE MECHANISM OF RECOVERY FROM LOBAR PNEUMONIA.

By RICHARD H. P. SIA, M.D., OSWALD H. ROBERTSON, M.D., AND SHUTAI T. WOO, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research, New York, and the Department of Medicine, Peking Union Medical College, Peking, China.)

Investigations into the mechanism of recovery from lobar pneumonia of recent years have brought forward an increasing amount of data to show that the principal evidence of acquired immunity consists in the development of humoral immune substances at or about the time of crisis. Yet the majority of authors writing on this subject express considerable caution in drawing conclusions as to the causal relation between the immune bodies and the recovery process even though certain of these substances or reactions have been shown to be directly concerned in the destruction of pneumococci and protection of the body from invasion. The reasons for this reservation of judgment are principally as follows: First, the occurrence of immune substances in the serum has not been observed as a constant phenomenon in all cases recovering from pneumonia; second, the appearance of these immune bodies, while generally coinciding with the onset of recovery, may occur 1 or 2 days before crisis or in some cases not until afterwards; third, serum immunity has been detected infrequently in cases terminating fatally; fourth, while the promotion of phagocytosis and intracellular destruction has been found to be one of the chief manifestations of acquired antipneumococcus serum action, the evidence for pneumococcidal power of the blood at the time of recovery is not generally considered as convincing. Furthermore, doubt has been cast on the importance of phagocytosis as a factor in the disposal of pneumococci in the body by the failure to demonstrate any degree of engulfment of these microorganisms by the cells of the lung exudate secured from patients during the period of recovery.

Finally, the fact that the introduction of large quantities of immune serum containing a high concentration of specific antibodies has been found by a number of investigators to be ineffective in cases of pneumonia after the 2nd or 3rd day of the disease, raises the question as to the ultimate rôle played by these serum substances.

The above considerations have led certain workers to seek other explanations for the sudden termination of infection in lobar pneumonia. Changes in the H ion concentration of pneumonic exudate in relation to the acid death-point of pneumococci have been studied (1). Investigation of the mobilization of enzymes in the blood at the time of crisis (2) have also brought to light interesting facts but they do not afford a satisfactory interpretation of the recovery process.

Our findings in a study of experimental pneumococcus infection in cats and rabbits (3) suggested that it would be profitable to pursue further by the same methods the inquiry as to the relation of acquired humoral immunity to the mechanism of recovery from lobar pneumonia in man. It was found that at the time of recovery from experimental disease there appeared constantly in the serum, immune properties characterized not only by opsonic, agglutinative, and mouse-protective activity, but also by the power to promote to a marked degree the killing of virulent pneumococci in rabbit serum-leucocyte mixtures. None of these changes occurred in animals with fatal infection. The degree of pneumococcus-killing power potential in the recovered animal's blood made it appear that this action played an important part in the disposal of pneumococci. While the development of humoral immune bodies was usually more marked and could be more closely related to the onset of recovery in the pneumococcus-resistant animal (the cat) than in the susceptible animal (the rabbit), yet the latter showed the same type of serum immune change and hence it seemed probable that in the human, whose resistance lies somewhere between that of these two animals, a similar immune reaction occurs.

Methods.

The methods employed in carrying out the pneumococcidal, opsonic, and agglutinative tests have been described in previous publications (4-6). The pneumococcidal-promoting power of the serum was tested by adding it in progressive dilution to rabbit serum-leucocyte mixtures containing small quantities of

TABLE I.

Determination of Pneumococcidal-Promoting Power of Serum during Course of Lobar Pneumonia, Pneumococcus Type II.

Human serum dilution 0.1 cc. + normal rabbit serum 0.2 cc. + rabbit leucocyte suspension 0.1 cc. + pneumococcus suspension 0.1 cc.

Serum sample in relation to lysis	Amount of standard pneumococcus suspension	Dilutions human serum	Growth as shown by color changes at hrs.*			Pneumococci in stained film at 72 hrs.
			16	48	72	
2 days before	10^{-6}**	1–10	++++			. +
	"	1–20	++++			+
1 day before	"	1–10	++++			+
	"	1–20	++++			+
Day beginning	"	1–10	0	0	0	0
	"	1–20	0	++++		+
	"	1–40	++++			+
	"	1–80	++++			+
	"	1–160	++++			+
Day completed	"	1–40	0	0	0	0
	"	1–80	0	0	0	0
	"	1–160	0	0	0	0
	"	1–320	0	0	0	0
	"	1–640	++++			+
	"	1–1280	++++			+
3 days after	"	1–40	0	0	0	0
	"	1–80	0	0	0	0
	"	1–160	0	0	0	0
	"	1–320	0	0	0	0
	"	1–640	++++			+
	"	1–1280	++++			+
12 days after	"	1–20	0	0	0	0
	"	1–40	++++			+
	"	1–80	++++			+
	"	1–160	++++			+
	"	1–320	++++			+
	"	1–640	++++			+
Controls	10^{-7}	0	++++			+
	"	0	++++			+

* Degrees of methemoglobin formation.

** This amount represents 500 to 1000 pairs of pneumococci.

highly virulent pneumococci. Since these mixtures have no growth-inhibitory action on such organisms, any pneumococcidal effect which occurs may be attributed directly to the added test serum. The amounts of the various constituents used in the reaction are given in Table I. Opsonic and agglutinative tests were performed by mixing pneumococci which had completed their active growth phase[1] with serum, in a ratio of 1 part of pneumococcus suspension to 20 parts of serum. After sensitization for 1 hour the pneumococci were then separated from the serum by centrifugation, the sedimented mass broken up with a capillary pipette, macroscopic agglutination noted, and phagocytic tests performed in the usual way with especially prepared rabbit leucocytes. The degrees of opsonic action and agglutination were noted in terms of plus signs ranging from $+$ slight but definite, to $+ + + +$ which was very marked. Since all the specimens of serum from one patient, inactivated after separation and kept on ice, were tested at one time it was possible to make comparative readings on them. The different tests, *pneumococcidal, opsonic, agglutination, and mouse protection*, were usually made on the same day. Occasionally this was not possible.

The pneumococci used in the tests with Type I sera were highly virulent strains. The homologous organisms isolated from both blood and sputum as well as a stock culture were used. In the tests with serum from pneumonia Type II and Group IV it was necessary to use the serum and leucocytes of young rabbits as none of the strains from these patients were virulent, nor could they be made virulent for full grown rabbits (7). Controls with young rabbit serum and leucocytes showed no opsonic or agglutinative action for these Type II and Group IV strains of pneumococci which were highly virulent for the young animal.

Clinical Cases.

The following study represents observations made on seventeen cases of lobar pneumonia comprising five cases of Pneumococcus Type I, six of Type II, two of Type II atypical, and four Group IV. Sixteen of the patients recovered. One died.

It may be stated briefly at the outset that the results of the study in human cases were essentially the same as those obtained in cats with experimental pneumococcus infection. Furthermore no constant differences in reaction were observed between the three types of pneumonia studied. The blood serum in the early stages of the disease was found to be without pneumococcus immune principles. Then, about the time of crisis, the serum acquired demonstrable

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[1] Type II pneumococci, however, were used in the active growth phase. The significance of the growth phase in relation to opsonic and agglutination tests is discussed in an earlier publication (*J. Exp. Med.*, 1927, xlvi, 239).
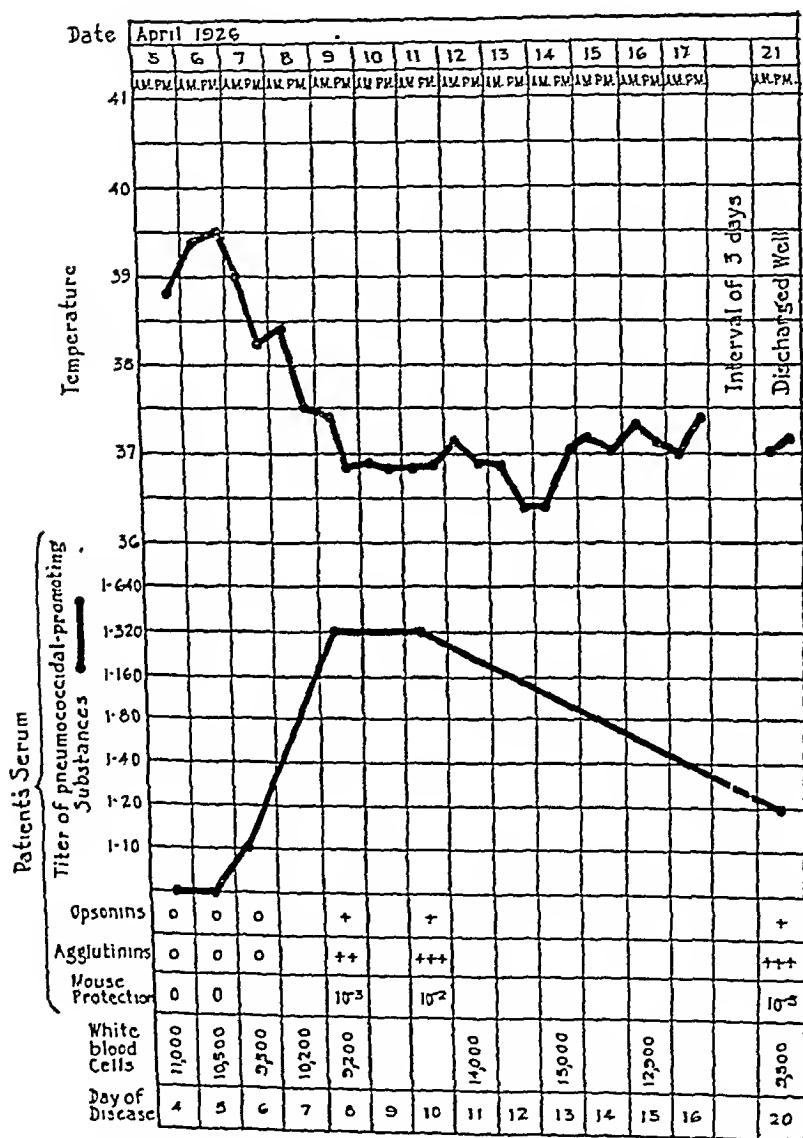
TEXT-FIG. 1. Case 13455. Lobar pneumonia, Pneumococcus Type II.

pneumococcidal-promoting power as well as opsonic, agglutinative, and mouse-protective properties. Text-fig. 1 illustrates well the sequence of changes found to occur in cases where the observations were begun early in the disease course. Tests on the blood specimens taken from this patient on the 4th and 5th days of disease were negative (Table I). On the 6th day, which marked the beginning of the fall in temperature, the serum showed the titer of pneumococcidal-promoting substances of 1:10. Opsonins and agglutinins were not demonstrable at this time. On the 8th day when the temperature had reached normal the titer of pneumococcidal-promoting substances had risen to 1:320 and opsonins, agglutinins, and mouse-protective bodies were evident.

The occurrence of demonstrable immune substances in the blood as shown especially by the pneumococcidal-promoting power, appeared to coincide generally with the beginning of defervescence. In only one instance (Text-fig. 4) were immune properties found in the serum more than a very short time (hours) before the temperature began to fall. In one other case pneumococcidal-promoting substances failed to appear until after the temperature had reached normal. It is quite possible that a more complete series of observations made immediately preceding the initiation of recovery might show a constantly earlier appearance of serum immune properties, although the results of tests such as are exhibited in Text-figs. 1 and 3, in which the immune reactions were found to be present initially to only a slight degree, suggest that they had not been detectable much earlier. In support of this supposition is the fact that once this immune property appeared in the blood it showed in most instances a rapid quantitative increase.[2]

The titer of pneumococcidal-promoting substances usually reached its height within 48 to 72 hours. Then after some days at this high level it began to fall. Just how long the serum continued to show immune properties was not determined but that the persistence of passive immunity varies greatly from patient to patient was shown by the finding in some cases that all traces of humoral immunity had

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[2] Our observations in experimental animals (3) in which a rapidly diminishing blood invasion occurred 24 to 48 hours before the termination of the disease make it seem highly probable that immune bodies are elaborated and perhaps present in low concentration for some time before crisis.
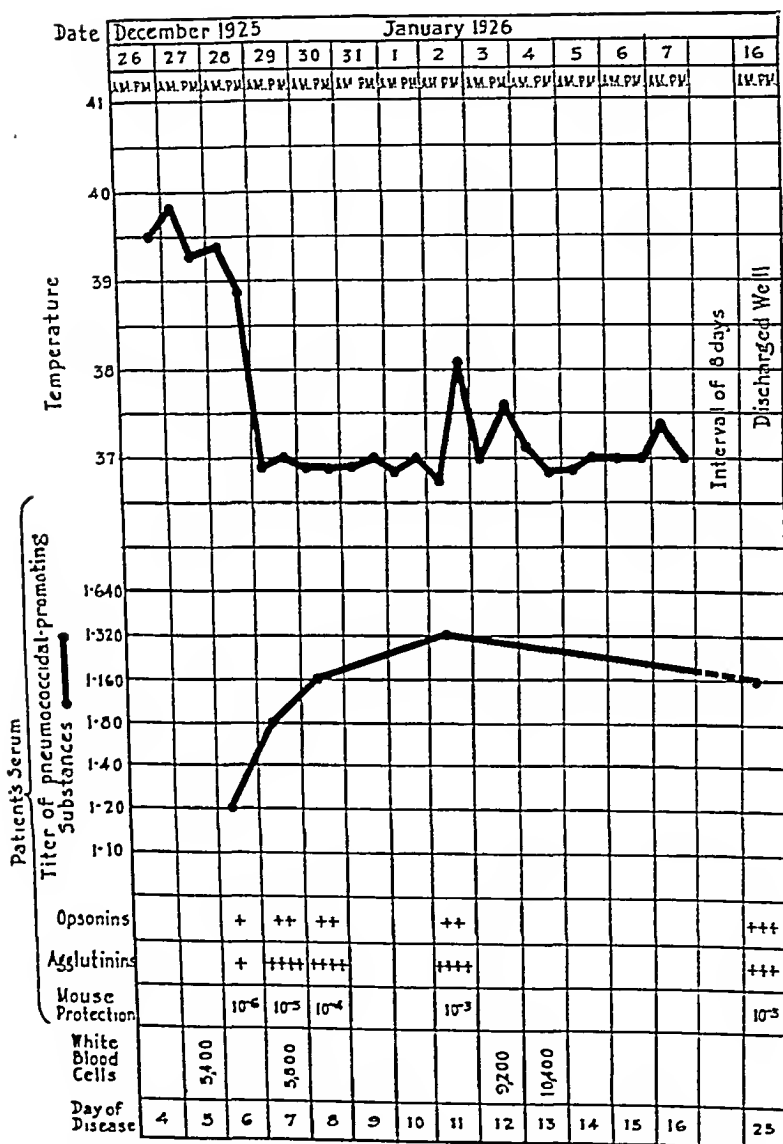
TEXT-FIG. 2.   Case 12656.   Lobar pneumonia, Pneumococcus Type II.
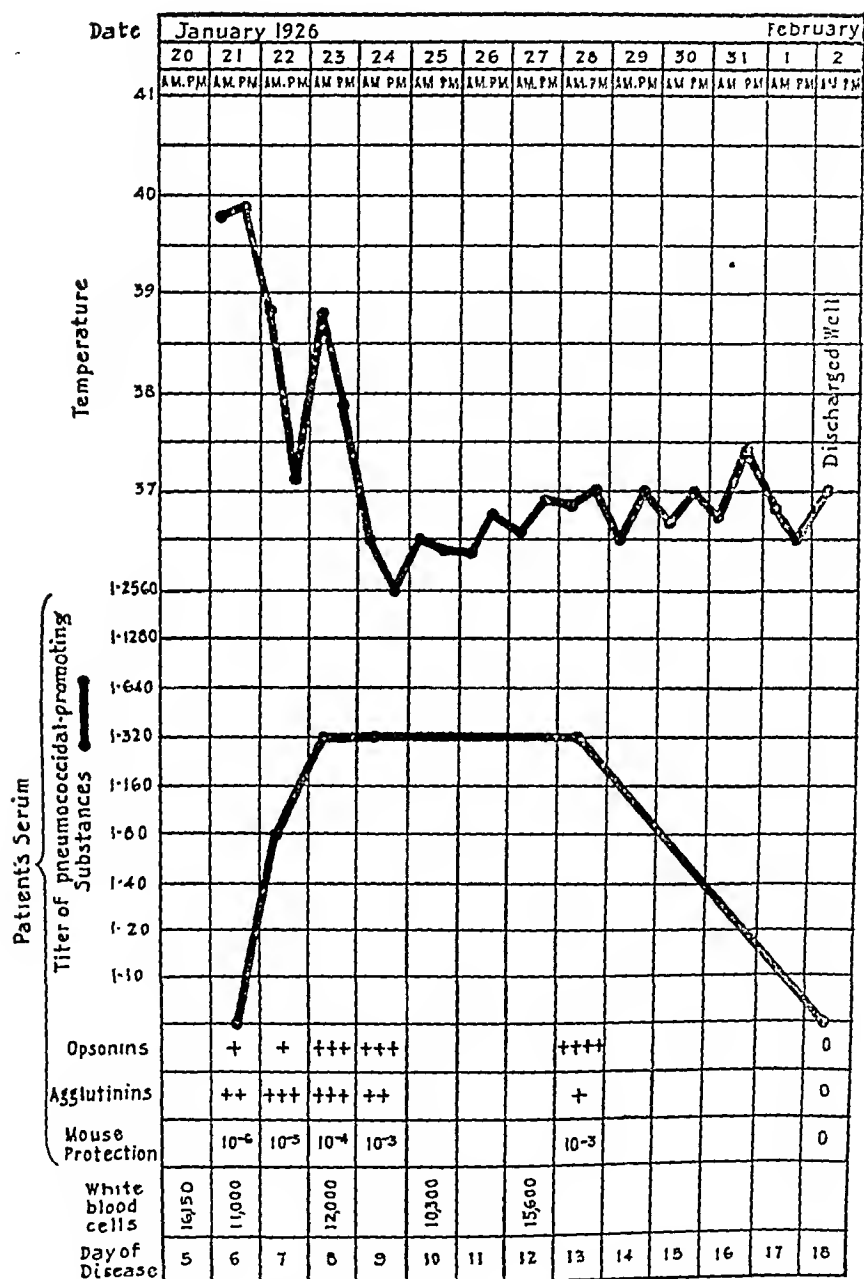
TEXT-FIG. 3.   Case 12827.   Lobar pneumonia, Pneumococcus Type I.
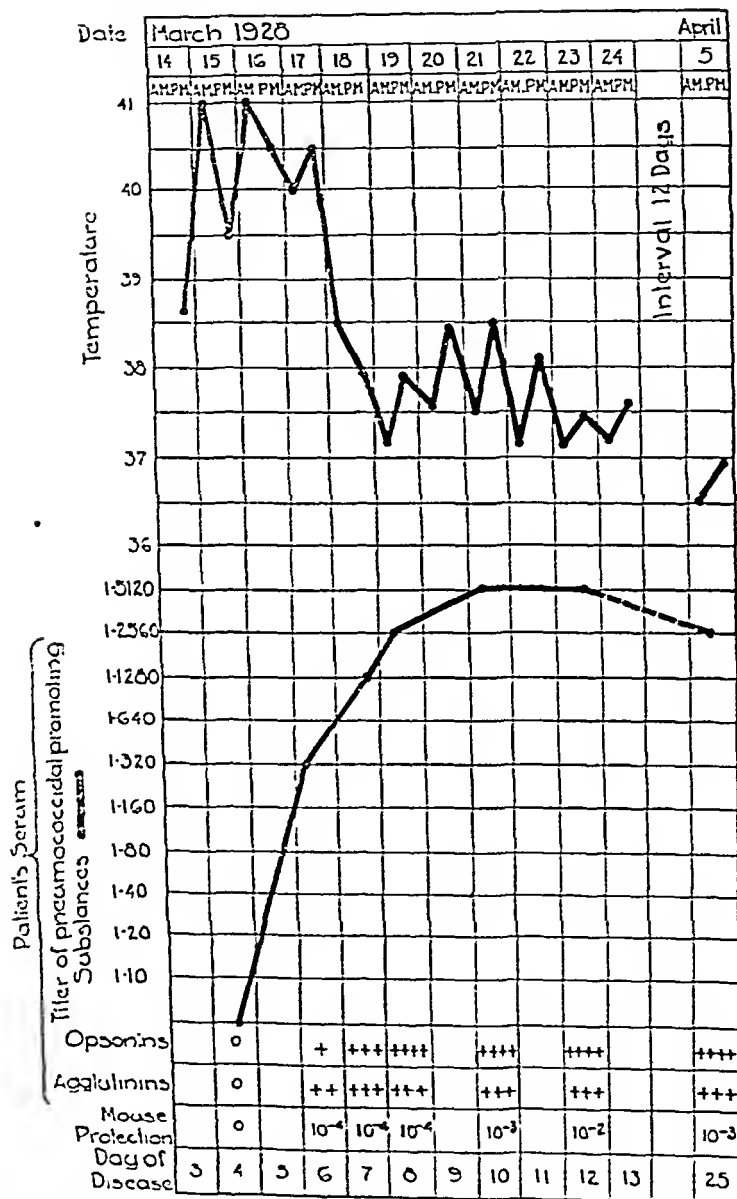
TEXT-FIG. 4.   Case 3193.   Lobar pneumonia, Pneumococcus Type II atypical.
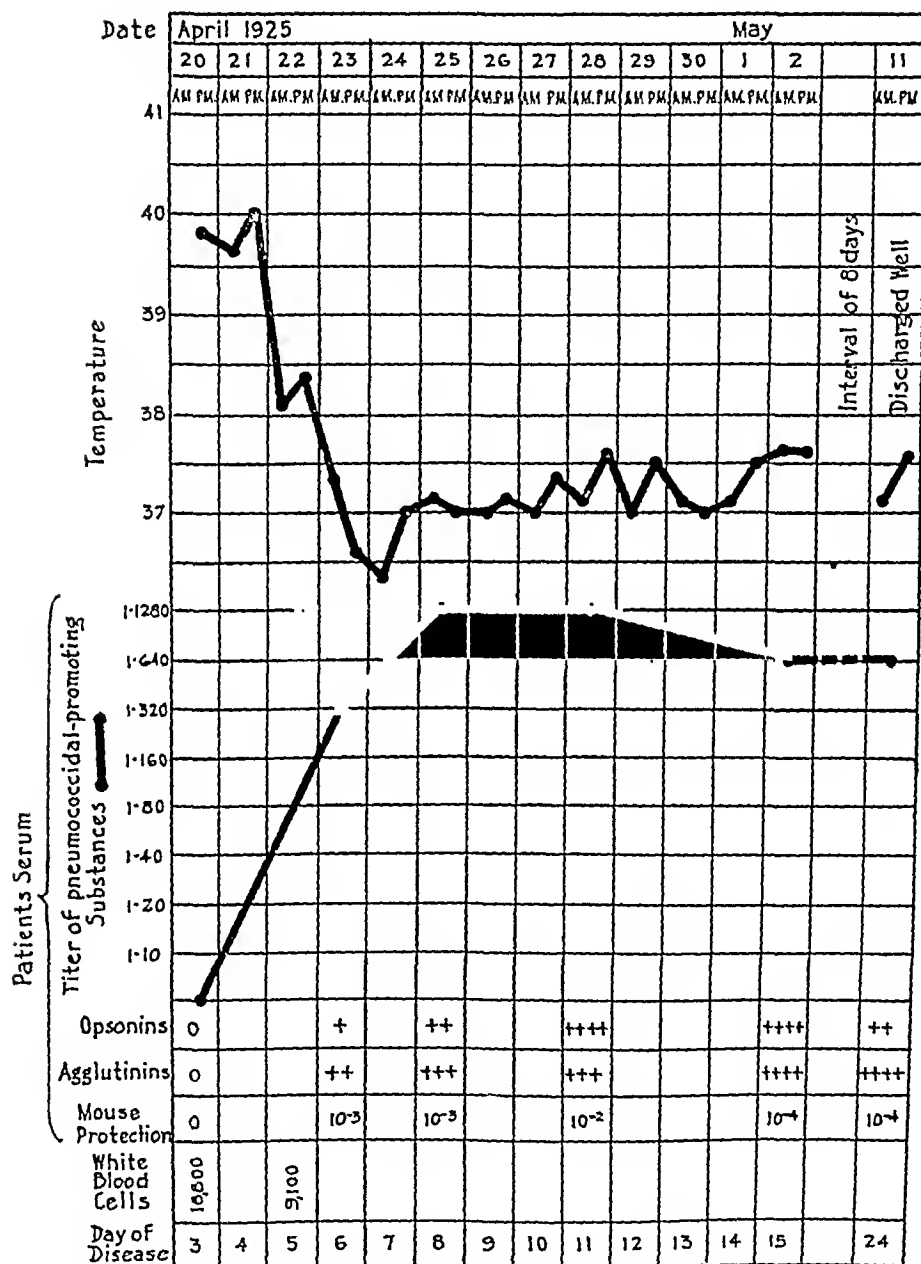
TEXT-FIG. 5. Case 10608. Lobar pneumonia, Pneumococcus Type II.

disappeared within 10 days after crisis (Case 12827, Text-fig. 3), while in others, and these constituted the majority, at the end of weeks or months serum immune properties were· still evident though much diminished.   Note Case 4960, Text-fig. 7, tested after 74 days.   The persistence of immune substances in the serum seemed to be associated to a certain degree with the initial intensity of immune body production.   Thus, those patients in whom serum immunity was demonstrable for the longest periods showed a relatively high pneumococcidal-promoting serum titer at outset, and those in whom the serum immunity disappeared earlier showed an initial low or at most moderately elevated titer.   However, these observations are too few to permit any conclusions concerning this point.

Only one patient of the sixteen recovering showed a positive blood culture.   This was taken on the day before the appearance of serum immune substances which occurred at the time of crisis, hence it was not possible in this series of cases to determine whether the occurrence of detectable pneumococcidal-promoting substances in the blood marked the termination of blood invasion as was always found to be the case in cats recovering from experimental pneumococcus infection.

In the majority of the cases all the different manifestations of serum immunity became demonstrable at the same time as in Cases 12656 and 3193, Text-figs. 2 and 4.   In others, the appearance of pneumococcidal-promoting power in the serum seemed to precede or occasionally lag behind the occurrence of opsonic, agglutinative, and mouse-protective action (Text-figs. 1, 3, and 6).   These variations may, however, be more apparent than real since the different means employed to bring out the several reactions may not be strictly comparable in their power to detect minute traces of the immune substance or substances.   Furthermore, a comparison of the changing intensity of the four reactions through the period of observation revealed a general parallelism.   This was closest between the pneumococcidal-promoting power and the mouse-protective action which are more susceptible to accurate quantitative estimation than are the opsonic and agglutinative reactions as carried out in this work.   It was found frequently, especially at the beginning of recovery, that equal parts of serum and pneumococcus suspension failed to bring out opsonic activity while employing 20 parts of serum to 1 of suspension produced a well defined reaction.
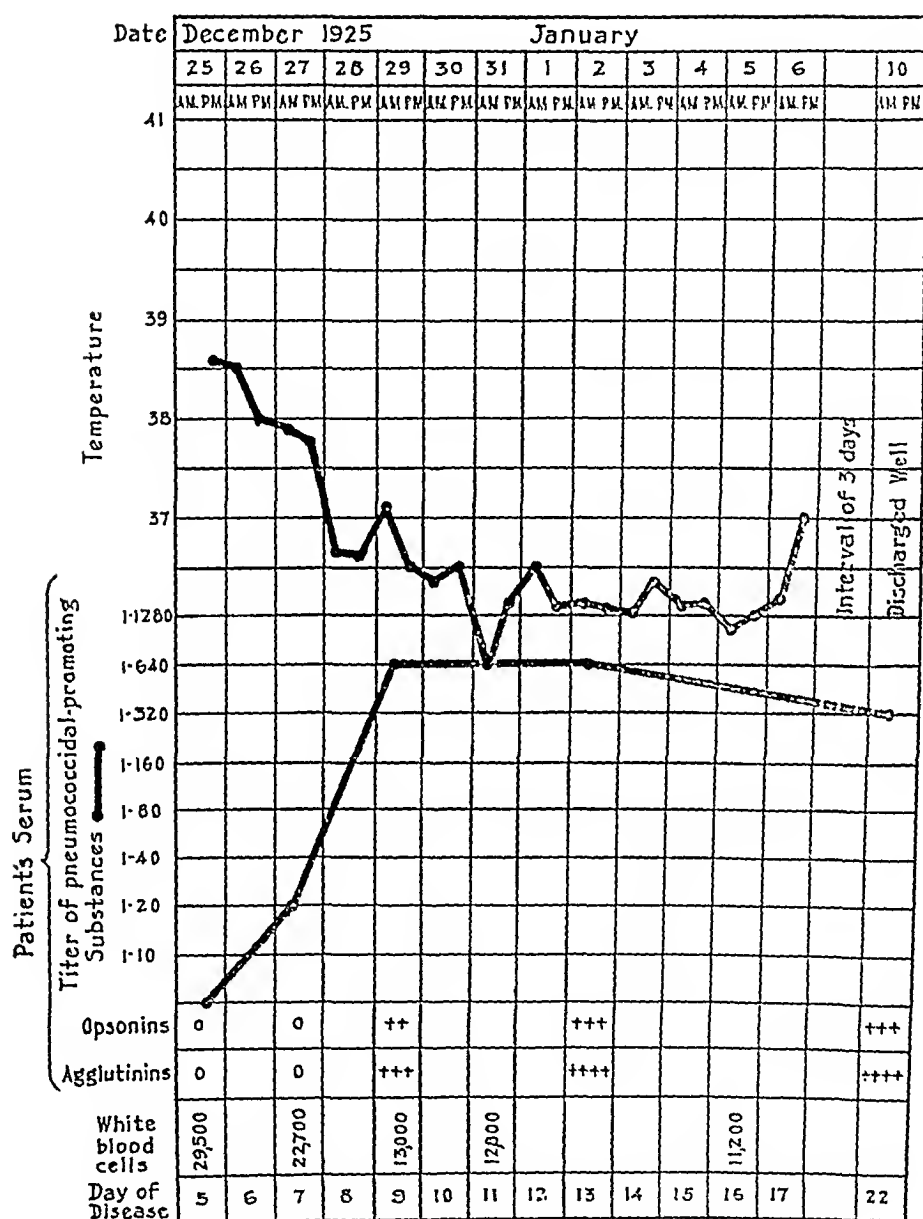
TEXT-FIG. 6.   Case 7797.   Lobar pneumonia, Pneumococcus Type II.

In striking contrast to the findings in patients recovering, are those made on the case progressing to a fatal termination (Text-fig. 8). Repeated tests of the blood serum made during the course of the disease in this patient failed to reveal any evidence of the development of immune properties. Tests for opsonic, agglutinative, and mouse-protective action were not made in this case but judging from the findings in experimental pneumococcus infection (3), it is probable that these reactions were also lacking here. There was a persistent slight blood invasion but it did not increase during the course of the disease.

Specificity of Immune Substances.

The pneumococcidal-promoting substances occurring in the serum at recovery were found to be strictly type-specific. Pneumococcidal tests in which one serum was used with several types of pneumococci and also several types of serum with one type of pneumococcus, showed killing of the pneumococci only in those tubes containing the homologous serum and organisms. The opsonic and agglutinative reactions were also found to be specific to type.

White Blood Cells.

The observations on the leucocytic response in this series of pneumonia patients are much less complete than those made in the experimental infected animals described in the preceding paper to which reference has been made (3). However, certain points are apparent and these are in general similar to the findings in the experimental disease. In the majority of the human cases there was a moderately increased or high white count just before the onset of recovery. In others, however, the number of leucocytes remained about normal and in one instance slightly below normal during the critical period (see Text-figs. 1 and 2). The fatal case showed an increasing white count towards the end of the disease reaching 35,000 the day before death.[3] These findings are in agreement with the inferences drawn from the animal studies, namely, that the leucocytes probably play a secondary rôle in the mechanism of recovery from pneumococcus infection, but

[3] There was no evidence of complications in this patient although this could not be definitely determined since an autopsy was not obtained.
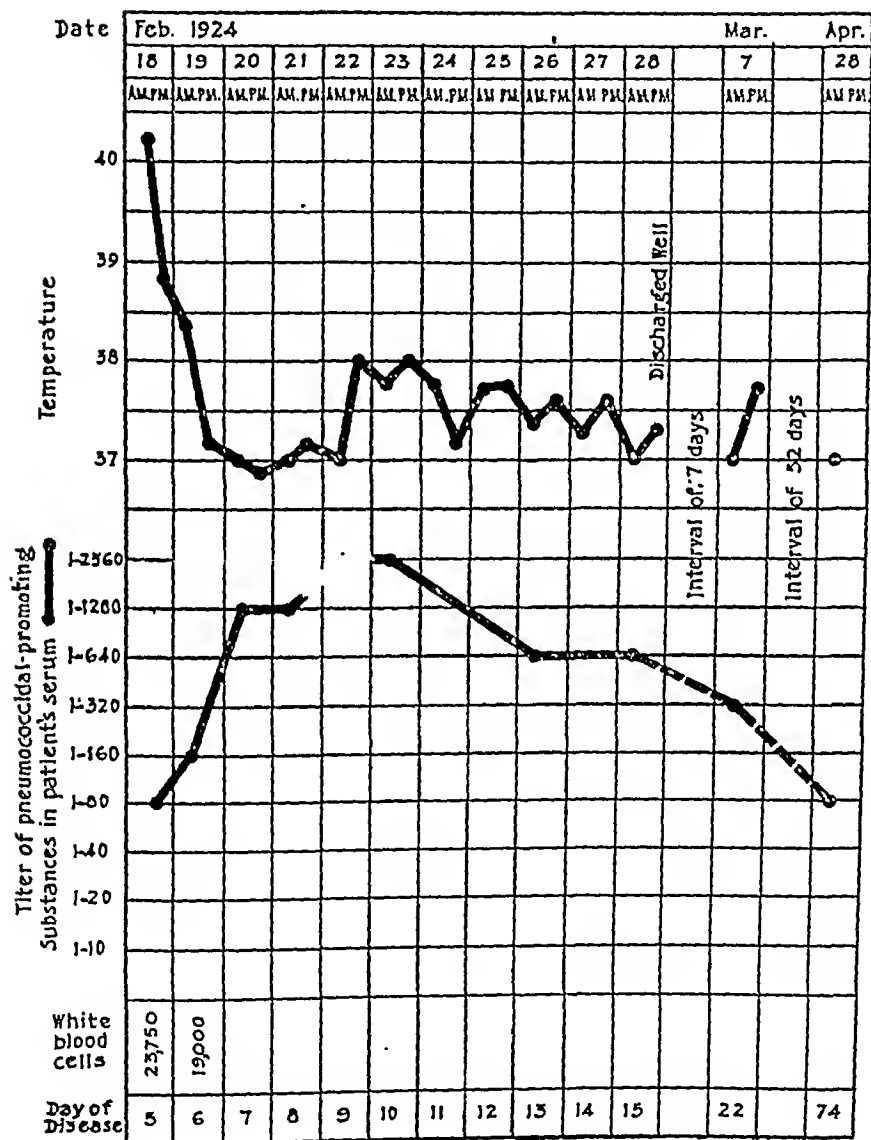
TEXT-FIG. 7.   Case 4960.   Lobar pneumonia, Pneumococcus Type I.
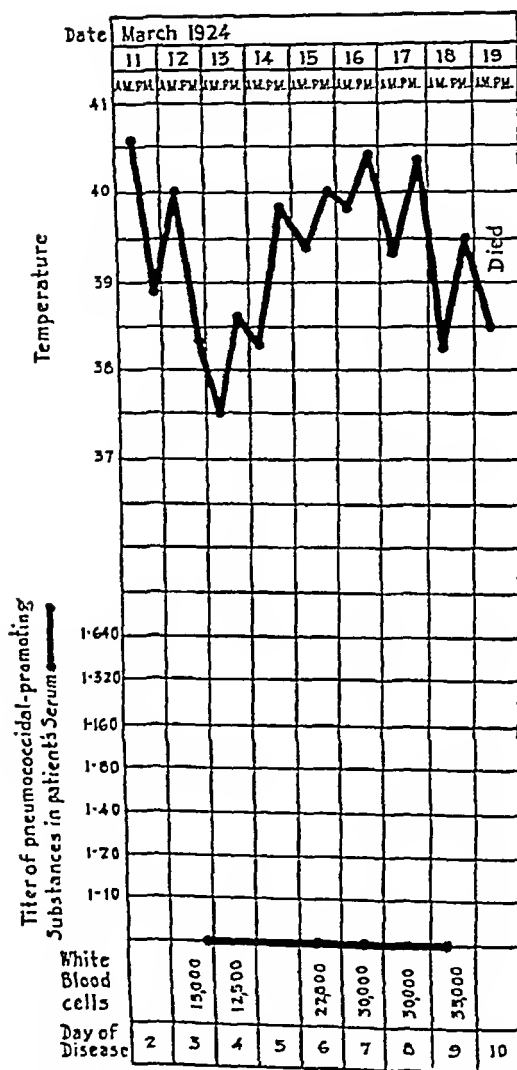
TEXT-FIG. 8.   Case 4983.   Lobar pneumonia, Pneumococcus Type II.

the well substantiated clinical observation that a low white count is generally to be considered as of unfavorable prognostic import, indicates that a certain minimum number of white blood cells are necessary to provide suitable conditions for recovery.

Complications and the Presence of Immune Substances.

Two of the patients in this series, a Group IV and a Type II pneumococcus pneumonia, developed empyema at a time when there were demonstrable pneumococcidal-promoting properties in the serum. In one instance the titer was low, 1:40, in the other it was 1:640. Both patients recovered.

DISCUSSION.

While the above findings do not warrant the conclusion that the development of specific antipneumococcus properties occurs constantly in the serum of all patients recovering from lobar pneumonia, they do indicate that these serum changes are of very common occurrence and perhaps are present to varying degrees in every case. The fact that certain workers, Chickering (8), Clough (9), Lister (10), and others, have failed to demonstrate the presence of opsonins and agglutinins in a small per cent of pneumonia cases at the time of crisis may simply indicate the occurrence of considerable variation in the degree of antibody response. We have shown that the use of equal parts of serum and pneumococcus suspension employed in the earlier studies may fail to bring out a low concentration of opsonins and agglutinins. Chickering mentions that in some of his cases agglutinins were detected on 1 day only. However, the absence of mouse-protective action in the critical and postcritical serum observed by certain investigators in occasional cases recovering from lobar pneumonia is of greater significance on account of the marked sensitiveness of this test.[4] Dochez (11) found in a series of ten recovering cases that the serum of one failed to show protective power against the homologous organism. Clough (12) studying twelve cases found protective action in the serum of only nine. In the three patients in whom the serum showed no protection, only one serum specimen was obtained in each case and this, 1 to 3 days following recovery. Other authors, and most recently

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[4] Mouse protection seems to be fully as delicate an indication of the presence of antipneumococcus immune substances as is the test for pneumococcidal action.

Baldwin and Rhoades (13), have found protective substances constantly in the serum of recovering patients.

Previous studies by other investigators have shown that in the majority of cases the appearance of circulating immune bodies coincides closely with the time of crisis or lysis. But in a small percentage of pneumonia patients immune substances have been detected 1 to 2 days beforehand. Baldwin and Rhoades (13) found protective bodies in twelve out of twenty cases at least 2 days prior to the day of crisis. However, a number of their reported cases had received specific therapy of one kind or another so that it is not clear how many of them developed the passive immune properties spontaneously. Does this occasional finding of immune properties in the serum sometime before crisis constitute a serious objection to the assumption that immune body development is related intimately to the mechanism of recovery? While the relationship between demonstrable humoral immunity and recovery would be much clearer if serum immune substances always appeared at or immediately before crisis, the fact that they may be present in the blood a day or two before the apparent beginning termination of the disease process does not seem to provide a sufficient reason for denying them an important rôle in the recovery mechanism. We know relatively little about conditions in the body which influence the functioning of opsonins, protective bodies, etc. Furthermore, structural peculiarities in the local lesion may well produce variation in the rate at which the immune substances penetrate or there may be other factors of a more general nature upon which the effective action of these antipneumococcus bodies largely depends. May not the early appearance of acquired immune bodies in a given case of lobar pneumonia represent a condition somewhat analogous to that which is frequently observed after the introduction of antipneumococcus serum Type I? Following the injection of an adequate amount of immune serum the blood acquires immune properties but the crisis may not occur for several days. However, the blood invasion ceases and the pneumonic process stops spreading. It has been shown by Baldwin and Cecil (14) that the appearance of protective bodies in the blood of spontaneously recovering cases marks the termination of blood invasion, a finding with which our observations in experimental pneumococcus infection agree. It is not possible that the spread of the pneumonic process

also ceases at this time? Our one patient in whom humoral immunity was detected 12 to 18 hours before crisis (Text-fig. 4) did show physical signs of beginning resolution 24 hours before the critical fall in temperature. We have found no observations on this point in the literature.

There exists also the possibility that the termination of the disease depends on the development of antitoxic as well as antibacterial immunity, as was suggested years ago by Cole (15), Wadsworth (16), and others.

Failure to detect immune substances until 24 hours or more after crisis has been reported less frequently than their early appearance. This condition might be due to an unusually marked neutralization of perhaps a rather low concentration of these bodies at the time of recovery.

The finding of antipneumococcus immune properties in the serum of patients going on to a fatal termination has been confined as far as we can determine to cases developing complications. In Baldwin and Cecil's (14) three fatal cases showing circulating humoral immune substances there was a complicating empyema. In Clough's (9) one reported case, an active endocarditis was probably present. The development of such secondary foci of infection in patients in whom the pneumonic process has been checked and who show evidence of considerable serum immunity is a further indication of the complexity of the recovery mechanism.

Of even more importance than the finding of antipneumococcus substances in the serum during recovery is information as to their mode of action. The demonstration in this study that a serum showing such properties possesses the power to promote the destruction of highly virulent pneumococci provides more direct evidence of the function of these immune bodies than has heretofore been obtained. That immune serum promotes phagocytosis and intracellular digestion in the body as it does in the test-tube seems highly probable although it should be pointed out that we have made no determinations of the activity of the pneumonic leucocytes. Investigations in this field by other workers (17–19) have shown that the leucocytes apparently function actively during the height of the disease.[5]

[5] A discussion of this phase of the subject is taken up in an earlier publication (3).

The fact that the above findings in human cases of lobar pneumonia are in practically entire accord with the observations made in cats undergoing experimental pneumococcus infection enhances considerably the significance of the development of serum immune bodies at the time of crisis.   In the cat it was possible to show that the acquisition of humoral immunity was associated with greatly increased antipneumococcus resistance.   Furthermore variations in the degree of acquired immunity were found to be associated in a general way with the corresponding fluctuations in concentration of demonstrable serum immune bodies.   But while these observations suggest that the reaction of the body to pneumococcus invasion is the same in animals and in man despite the great dissimilarity in the character of the lesions produced, they do not indicate certainly that the mechanism of recovery is identical in both instances.   The peculiar character of the local lesion in lobar pneumonia would seem to introduce certain special conditions to be reckoned with in the human body's defense against this organism and which may involve the operation of processes other than those essential for recovery in less intensely localized pneumococcus infection.   In a discussion of this question, Cole (20) suggests that the destruction of pneumococci in the lung lesion may depend on local factors quite different from those responsible for the destruction of the bacteria in the circulating blood.   He mentions the marked changes which occur in the resolving lung exudate, the solution of fibrin acting as a possible relief from tension and affording an outlet for the exudate and the production of chemical compounds, soaps, fatty acids, etc., shown to have a destructive action on pneumococci.   However, he points out the difficulty in inferring that the crisis depends mainly on the resolution of the exudate, in that these two processes do not necessarily occur synchronously.   Cole's conclusion (21) that recovery depends largely on an adequate concentration of immune bodies in the blood is supported by the observations presented in this paper.

SUMMARY.

Employing a method devised for the investigation of natural immunity and experimental pneumococcus infection, a study has been made of the serum immune changes occurring during the course of

lobar pneumonia due to Pneumococcus Types I and II and Group IV, in man. It was found that at the time of crisis or lysis the blood serum acquired constantly the property of promoting pneumococcus killing to a relatively marked degree. Other evidences of antipneumococcus reaction—mouse protection, opsonins, and agglutinins—were also demonstrable in the blood at this time. These immune changes appeared in the majority of cases at the beginning of recovery and failed to occur when the disease terminated fatally. The fact that these observations in human cases are practically identical with previous findings in the experimental disease in cats, enhances considerably the significance of the development of serum immune bodies at the time of crisis since in the experimental animal it was possible to show that the acquisition of passive immunity was associated with greatly increased antipneumococcus resistance.

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16. Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 78.
17. Tunnicliff, R., *J. Infect. Dis.*, 1911, viii, 302.
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NITROGEN METABOLISM OF NORMAL AND SARCOMA-TOUS[1] FIBROBLASTS IN PURE CULTURES.

By LILLIAN E. BAKER, Ph.D., and ALEXIS CARREL, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

It has been reported that the proteoses of Witte's peptone and the primary split products of many different proteins. although not a complete nutritive medium, cause a rapid proliferation of the fibroblasts migrating from fresh embryo heart.[2,3]   Experiments on the fractionation of embryo juice, which promotes, as is well known, the unlimited growth of certain tissues *in vitro*, demonstrated that fibroblasts utilize the protein fraction.[4]   On the other hand, they proliferate for only a short time when fed upon the amino acids present in the embryo juice,[5] or formed by hydrolysis of the proteins.   Therefore, the hypothesis was proposed that enzymes present in the embryo juice, or within the cells, hydrolyze the proteins, and that the proteoses so formed determine the growth of the tissues.   In the experiments described in the present paper, an attempt was made to ascertain whether some one fraction of the proteoses, or the proteoses as a whole, are responsible for growth and also whether other fragments of the protein molecule, such as peptones, peptides, and amino acids, contribute to cell nutrition.

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[1] The sarcomatous fibroblasts referred to in this paper are from a pure strain of malignant fibroblasts from rat Sarcoma 10 of the Crocker Foundation, which have been cultivated *in vitro* for 18 months.

[2] Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503.

[3] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 353.

[4] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 387.

[5] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1926, xliv, 397.

Action of the Proteose Fractions on Normal and Sarcomatous[6] Fibroblasts.

Witte's peptone was separated into heteroproteose by dialysis, and alpha and beta proteoses[7] by alcoholic precipitation. The alcohol was removed by evaporation *in vacuo*, and the fractions were dissolved in Tyrode solution, or rendered isotonic by the addition of the salts of Ringer solution and tested at pH 7.4 on fibroblasts from chick embryo heart, and also on a pure strain of sarcomatous fibroblasts of the rat.[1] The heteroproteose proved to be practically inactive, but both the alpha and beta proteoses caused a rapid proliferation of normal and sarcomatous fibroblasts. When tested at equal nitrogen concentrations, both the alpha and the beta proteoses produced approximately the same amount of tissue as the entire peptone solution (Text-fig. 1). The cells cultivated in alpha proteose, however, remained in better condition than those in beta proteose or peptone, and showed fewer of the granulations which are characteristic of fibroblasts cultivated in the hydrolytic products of proteins. Since Witte's peptone and the beta fraction contain some of the lower fragments of the protein molecule, it seems probable that these fragments are more toxic than the alpha fraction. Purification of the alpha proteose by repeated precipitation did not decrease its growth-promoting power for either normal or sarcomatous fibroblasts (Text-figs. 2, 3). The precipitations were repeated until a constant nitrogen concentration in three successive filtrates indicated, as pointed out by Haslam,[8] that all other fractions were removed. Peptic digests of fibrin were also fractionated into a part precipitated by hydrochloric acid at pH 5.0 and another part soluble at this acidity. Both fractions contained proteoses and

[6] For the technique of cultivating the tissues and making comparative experiments on the nutritive action of different media, see: Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xliv, 503; Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371; Carrel, A., *Compt. rend. Soc. Biol.*, 1927, xcvi, 601. In these experiments, the plasma coagulum was washed for 30 minutes with 3 cc. of Tyrode solution, shortly after coagulation took place.

[7] Fractionation of the proteoses into the proto- and deutero-fractions was not made, since such fractionation necessitates the use of salts. In previous experiments on the fractionation of embryo juice, the use of these salts always resulted in toxic or inactive products. Even when the greatest care was used in removing the salts, sufficient traces seemed to be left to exert a harmful effect on the cells.

[8] Haslam, H. C., *J. Physiol.*, 1905, xxxii, 267; 1907, xxxvii, 164.
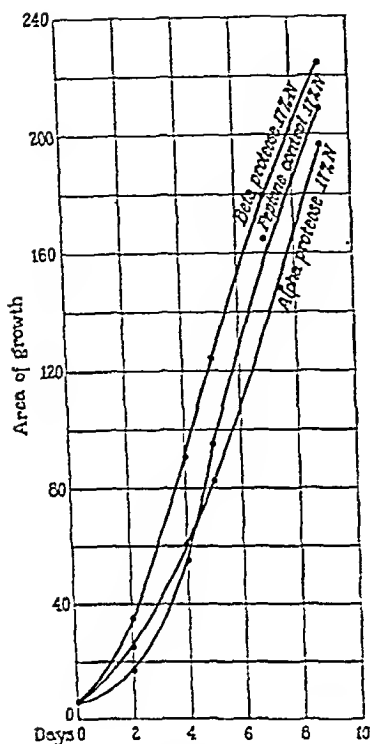
TEXT-FIG. 1.

TEXT-FIG. 2.

TEXT-FIG. 1. Experiment 9095-D. Comparative growth of fibroblasts from embryonic chick heart in alpha proteose, beta proteose,[9] and Witte's peptone at equal concentrations of nitrogen.
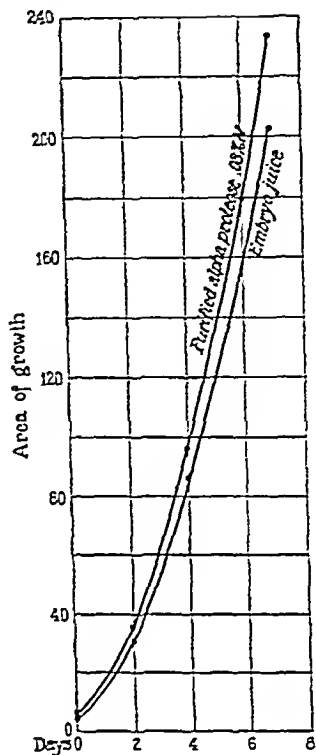
TEXT-FIG. 2. Experiment 9104-D. Comparative growth of fibroblasts from embryonic chick heart in purified alpha proteose,[9] and in embryo juice.

produced a large and approximately equal growth of sarcomatous and normal fibroblasts. It is evident, therefore, that the growth-promoting action of proteoses[9] is not a specific property of any one fraction,

[9] Although the proteoses produce a rapid growth for 8 or 10 days, they are not capable of supporting the life of the cells indefinitely. Other substances of unknown nature are required for the complete nutrition of the fibroblasts. A quantity of these substances sufficient for 8 or 10 days appears to be present already in the tissues. Salts and glucose are furnished by the Tyrode solution in which the proteoses are dissolved.

but that various fractions with different chemical properties are utilized by the cells.
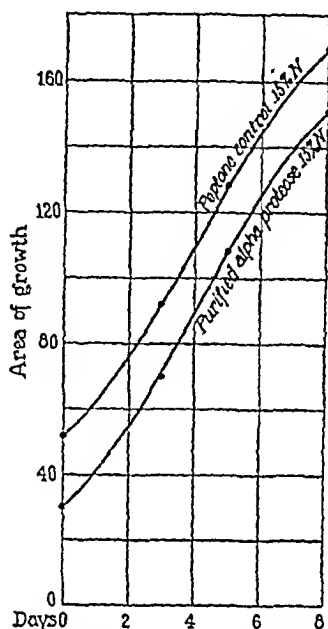
TEXT-FIG. 3. Experiment 6149–C. Comparative growth of sarcomatous fibroblasts of the rat in Witte's peptone and purified alpha proteose.[9]

Action of the Peptones and Lower Protein Hydrolytic Products on Normal and Sarcomatous Fibroblasts.

The first attempts to ascertain whether the peptones, peptides, and amino acids are utilized by the cells were unsuccessful. Isolation of these fractions from Witte's peptone or protein digests, involving the use of such substances as salts, phosphotungstic acid, etc., resulted in inactive or toxic preparations. To avoid the use of injurious chemicals, the mixture of proteolytic products obtained by prolonged tryptic digestion was tried. Some growth occurred, but the cells degenerated rapidly. It was impossible to know whether the rapid degeneration was due to a toxic action of the lower protein degradation products or to the absence of other substances required to supplement their action. Moreover, pure strains of cells in the presence of hydrolytic products of protein underwent some change which caused the fibrin clot imme-

diately surrounding the colony to liquefy or digest.   Thus, it seemed necessary to postpone further work on this subject until these difficulties could be overcome.   Recently, the discovery of two facts has rendered possible the continuation of this investigation: (1) Pure strains of normal and sarcomatous fibroblasts of the rat can be cultivated in chicken plasma, even in the presence of protein split products, without liquefaction of the fibrin clot.[10]   (2) Malignant fibroblasts from rat Sarcoma 10 of the Crocker Foundation will proliferate indefinitely in a peptic digest of liver.[11]   While the chemical nature of all the substances in these liver digests which render possible the unlimited life of sarcomatous tissue *in vitro* is not yet known, it seemed probable that the use of liver hydrolyzed to various degrees would show whether products other than proteoses could be utilized by sarcomatous fibroblasts.   Unfortunately, this method could not be applied as successfully to normal fibroblasts since, for some still unknown reason, they will not live indefinitely in the digests of liver.   They proliferate rapidly at first, but accumulate large quantities of fat, multiply more slowly, and finally die.[12]   In order that the substances of unknown nature which supplement the proteolytic products should not be removed, the various fractions were not isolated, but the total digest was used as a culture medium.   By hydrolyzing liver with pepsin, trypsin, erepsin, and hydrochloric acid, and by modifying the concentration of enzymes and the hydrogen ion concentration, products were obtained in which the amount of proteoses varied from 0 to 50 per cent, and that of the nitrogen present as free amino groups from 19 to 81 per cent.

These experiments revealed that it is not only proteoses which furnish the nitrogenous materials required for growth, but that the lower split products can support the indefinite multiplication of the malignant fibroblasts from Sarcoma 10.   While the destruction of proteoses by tryptic digestion decreased the growth of normal fibroblasts migrating from chick embryo heart,[2] the sarcomatous fibroblasts

[10] Carrel, A., *Compt. rend. Soc. biol.*, 1927, xcvi, 1119.

[11] Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1928, xlvii, 371.

[12] For comparison of the growth of normal and sarcomatous fibroblasts in liver digests, see Text-fig. 3 in Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1928, xlviii, 105.

of the rat were found to multiply as rapidly and for as long a period in the tryptic digest of liver as in the peptic digest.

Effect of the Hydrolytic Products of Liver on Sarcomatous Fibroblasts.

The hydrolytic products of liver may be classified in four groups according to their action on sarcomatous fibroblasts. All were used at a nitrogen concentration of .03 to .04 per cent.

1. Peptic Digests in Which the Ratio of Total to Amino Nitrogen Was Above 2.8.—These digests contained 19 to 35 per cent of their nitrogen in the form of free amino groups and 40 to 50 per cent as proteose. When these substances were used, the rate of growth was slow, much less than in embryo juice or in the liver digests previously described.[11] The cells were scattered, very large, round and flat, and filled with many fatty globules. In fact, they seemed to be degenerating cells, although they kept these characteristics and continued to multiply slowly for about 6 weeks.[13]

2. Peptic Digests in Which Digestion Was More Complete.—35 per cent or more of the nitrogen was present as free amino groups, and 33 per cent or less as proteose. In these digests, the sarcomatous fibroblasts proliferated indefinitely and the cells remained in normal condition, like those cultivated in embryo juice.[14]

3. Tryptic Digests Hydrolyzed as Completely as Possible.—In these digests, the proteoses were entirely destroyed.[15] The ratio of total to amino nitrogen was 1.45, and 69 per cent of the nitrogen was present as free amino groups. Attempts were made to hydrolyze the remain-

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[13] This conclusion was drawn from twenty-four experiments with seven different digests.

[14] This conclusion is the result of observations made during 1 year of colonies grown in many different preparations of this medium.

[15] When this preparation was tested for proteose by saturating at 33°C. with sodium sulfate, it was estimated that 3 per cent of proteose was present. However, since it is known that sodium and ammonium sulfate also bring down a small amount of the other proteolytic products, a control experiment was tried in which a mixture of pure amino acids and dipeptides was saturated with sodium sulfate at 33°C. Here also, 3 per cent of the nitrogen was precipitated. It is, therefore, concluded that this 3 per cent is not all proteose and, if any proteose remains, the quantity is negligible. At the dilution of this substance which was used in the culture medium, the quantity of proteose would be entirely without effect.

ing peptide linkings by further hydrolysis with enzymes, either trypsin or erepsin, but an equilibrium was always reached at which 13 per cent of the nitrogen remained in the peptide form. The remaining 18 per cent was in other forms which were not altered by boiling
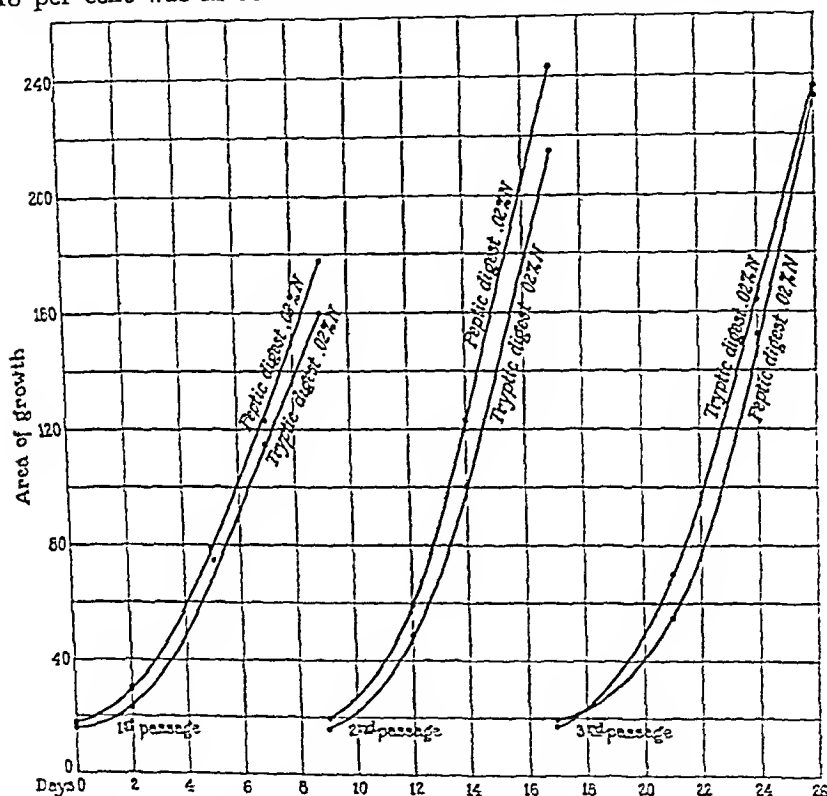
TEXT-FIG. 4. Experiments 3987-A, 4020-A, and 4040-A. Comparative growth of sarcomatous fibroblasts of the rat for 26 days in the products of the peptic hydrolysis and complete tryptic hydrolysis of liver at equal nitrogen concentrations. Every 8 or 9 days, the colony was cut in half and transferred to a new flask.

hydrochloric acid. In such media the sarcomatous fibroblasts proliferated indefinitely as rapidly as they did in embryo juice or in the peptic digests of Group 2 (Text-fig. 4). After the tissues had been cultivated for 102 days (Text-fig. 5), it was concluded that these preparations provided all the substances required for the growth of
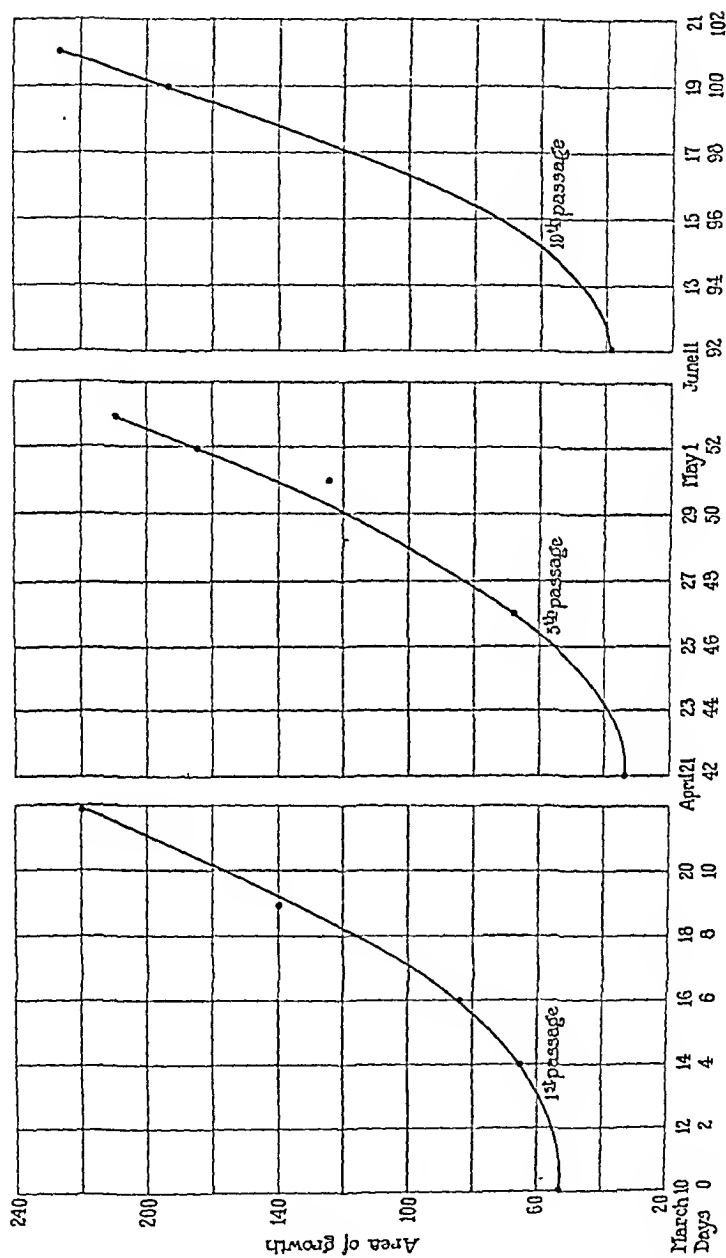
TEXT-FIG. 5. Experiments 4289-A, 4478-A, and 4663-A. Growth of sarcomatous fibroblasts of the rat in the first, fifth, and tenth passages, or for 102 days, in the tryptic digest of liver containing 13 per cent of peptide nitrogen. The rate of growth was uniform throughout all the intermediate passages.

sarcomatous fibroblasts.[16] The cells differed in their appearance from those cultivated in embryo juice or peptic digests. They were small and oval shaped, and often grew in a sheet reminding one of epithelium. At the same time, the cytoplasm was filled with small fat droplets and numerous granules, and appeared less transparent than that of the cells cultivated in embryo juice. When transferred to embryo juice, the fibroblasts quickly became transparent again and contained fewer granulations.

4. *Liver Completely Hydrolyzed by Boiling Hydrochloric Acid.*—No growth occurred in these preparations, even when tryptophane was
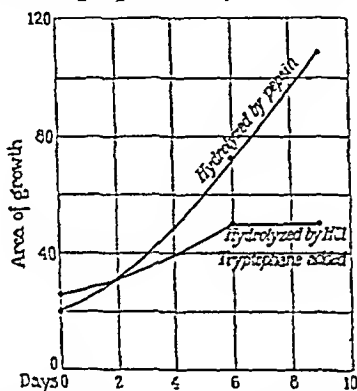
TEXT-FIG. 6. Experiment 4397-A. Comparatve growth of sarcomatous fibroblasts of the rat in the products of peptic hydrolysis of liver, and in the products of complete hydrolysis of liver by boiling hydrochloric acid. Tryptophane was added to replace that destroyed by the acid.

added to replace that destroyed by the acid (Text-fig. 6). These results are in accord with those of previously reported experiments[5] on the amino acids of embryo juice and artificial mixtures of amino acids. Although the amino acids present in the tryptic digests without doubt contribute to the growth of the tissue, and although glycocoll as such is utilized by them when added to albumin and casein digests, as shown in previous experiments,[3] amino acids alone have never been able to support cell proliferation. It appears that the 13 per cent of peptide nitrogen which remains in the tryptic digests is essential for the life of the cells *in vitro*.

[16] This conclusion was drawn from numerous experiments with four different preparations.

Several other preparations containing proteolytic products of liver have been tried. The results of these experiments agree with the preceding observations. Autolysis of liver at pH 1.5, the acidity used for preparing the peptic digests, resulted in a product of ratio 5:1, which resembled in its action the peptic digests insufficiently hydrolyzed. But autolysis at pH 4.5[17] gave a product of ratio 1:5, which caused rapid cell proliferation (Text-fig. 7), for a long period of time.
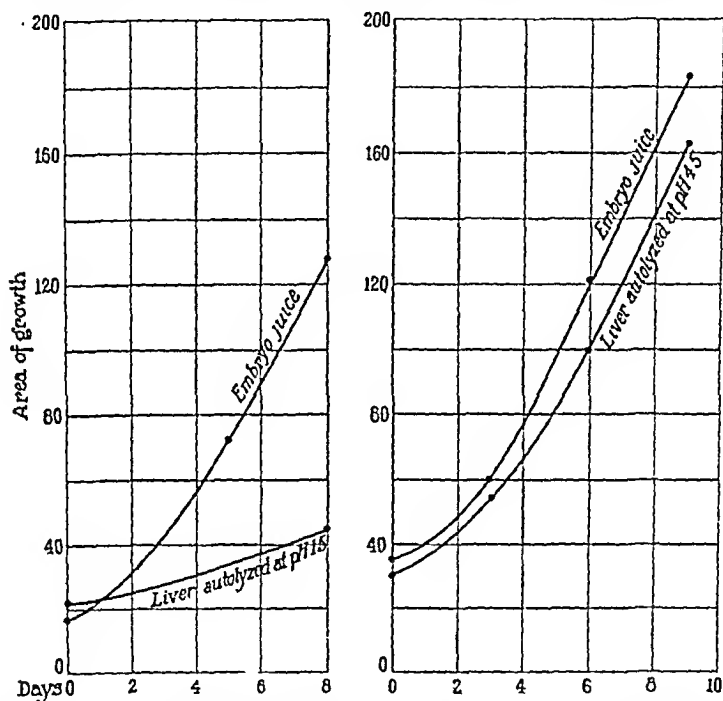
TEXT-FIG. 7. Experiments 4260-A and 4509-A. Comparative growth of sarcomatous fibroblasts of the rat in the products of liver autolysis at pH 1.5 and pH 4.5 with that in embryo juice.

The proteolytic products already present in liver were extracted with water at pH 9.0, and the proteins precipitated by adjusting to pH 5.0 and heating. This procedure was the same as the first steps taken by Cohn[18] in isolating from liver the substances effective in pernicious anemia. The resulting solution, like the second class of peptic

[17] A pH of 4.5 has been shown by Bradley to be the optimum for the activity of the autolytic enzymes of the liver. Bradley, H. C., *J. Biol. Chem.*, 1922, lii, 467.

[18] Cohn, E. J., Minot, G. R., Fulton, J. F., Ulrichs, H. F., Sargent, F. C., Weare, J. H., and Murphy, W. P., *J. Biol. Chem.*, 1927, lxxiv, p. lxix.

digests, had a ratio of total to amino nitrogen of 2:65, and proved capable of determining a prolonged growth of sarcomatous fibroblasts[19]
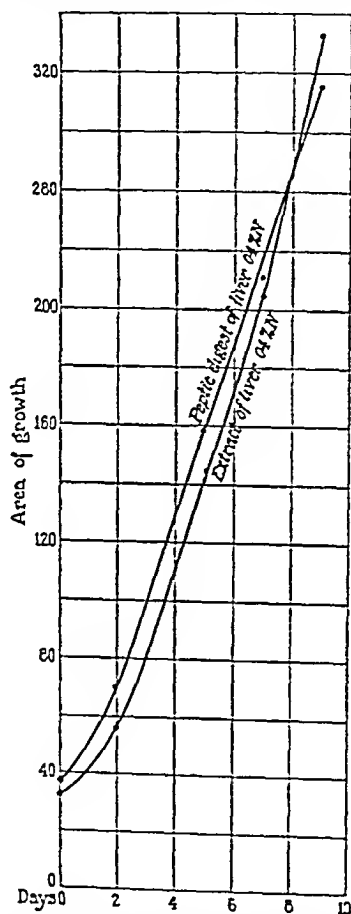
Text-Fig. 8. Experiment 10,173-D. Comparative growth of sarcomatous fibroblasts of the rat in Cohn's extract of liver and in the products of the peptic hydrolysis of liver at equal nitrogen concentration.

[19] The experiment was discontinued after 1 month. It is probably the proteolytic products already present in various organs which cause extracts of these organs to stimulate the multiplication of fibroblasts, as has been shown by Carrel, and Heaton. Carrel, A., *J. Exp. Med.*, 1913, xvii, 14; Heaton, T. B., *J. Path. and Bact.*, 1926, xxix, 293.

(Text-fig. 8). In fact, the autolytic products were in some respects better than the products of digestive enzymes, for the cells cultivated in such a medium were more transparent and similar to those maintained in embryo juice.

Fractionation with alcohol was tried in a few experiments. The constituents of a peptic digest soluble in 80 per cent alcohol produced for a considerable time as good a growth as the entire digest. The fraction soluble in 95 per cent alcohol failed to promote growth.

The above facts may be summarized as follows:

Products of the incomplete hydrolysis of the liver, either by pepsin or autolysis, that have less than 35 per cent of their nitrogen as free amino groups, do not support the growth of sarcomatous fibroblasts indefinitely. Peptic, autolytic, or tryptic digests, that have 35 to 69 per cent of their nitrogen as free amino groups, determine the unlimited proliferation of the cells. The active products are due to enzymatic action and are also preformed in the liver. Proteoses are utilized but are not required for growth. Peptones, peptides, and amino acids are also utilized. No growth, however, takes place in a medium in which all the peptide linkings are destroyed by acid hydrolysis. Although the acid may possibly act upon some other active group, there is little doubt that some peptides are required for the life of the cells.

Effects of the Hydrolytic Products of Liver on Normal Rat Fibroblasts.

Although normal cells do not proliferate indefinitely in liver digests, experiments of short duration were made with a view of comparing the behavior of normal and of sarcomatous fibroblasts. It was found that normal rat fibroblasts proliferated as rapidly for the first 5 or 6 days in the tryptic digests containing no proteose, as in the peptic digests. In both cases, the cells filled with fat, and growth decreased greatly in the second passage. The same was true in preparations of edestin and fibrin hydrolyzed to different degrees. It must be concluded, therefore, that normal cells also utilize the lower degradation products of protein. This does not contradict the previous observations[2] on the growth-activating effect of proteoses and

lower split fragments on fibroblasts from chick embryo heart, because pure strains of cells are more susceptible than fresh tissue to the toxic action or to the deficiencies of their medium, and because the hydrolytic products of liver used in these experiments, even those least hydrolyzed, always contained a large portion of the lower products. When these preparations were highly diluted so that they contained less than
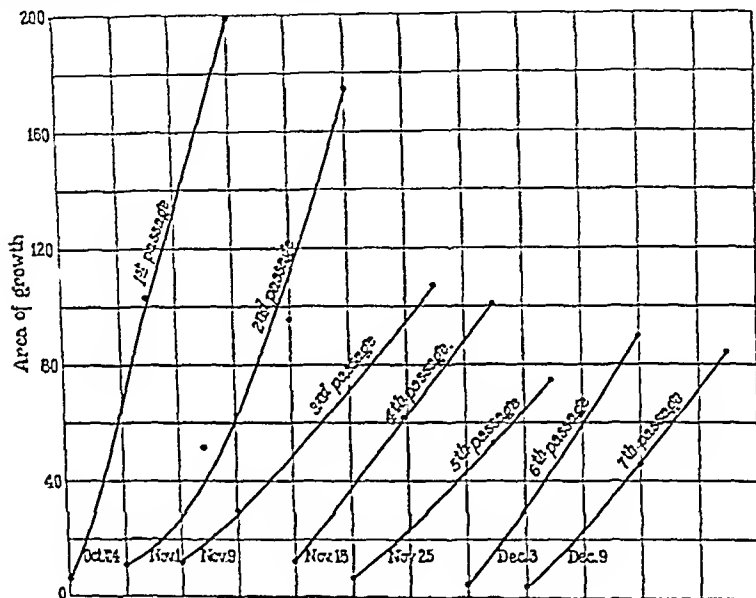
TEXT-FIG. 9. Experiment 3823-A. Growth of a pure strain of normal chicken fibroblasts in a peptic digest of liver. This digest was not boiled, but the toluene used during digestion was removed by aeration with nitrogen containing a small per cent of oxygen. Concentration of nitrogen, .02 per cent.

.02 per cent nitrogen, their toxic action was greatly reduced.[20] Normal fibroblasts could be kept alive in them for long periods of time without showing the characteristic, fatty degeneration deposits, and would multiply very slowly. In some of the proteolytic products of liver, pure strains of normal rat, and also of normal chicken, fibro-

[20] In the experiments previously reported, the concentration of proteolytic products in the media was much larger than that used in the present experiments. The large difference in growth shows that the lower fragments of the protein molecule are much more toxic to normal cells than the proteoses.

blasts were kept alive and growing very slowly for 2 months, at which time the experiment was discontinued (Text-figs. 9, 10). As the colonies had to be transferred to a new flask every 6 or 10 days, it is probable that some substance, possibly an enzyme present in the new plasma, supplemented the nutritive action of the liver digests.

The differences observed between the growth of the normal and sarcomatous rat fibroblasts in these media cannot as yet be explained.
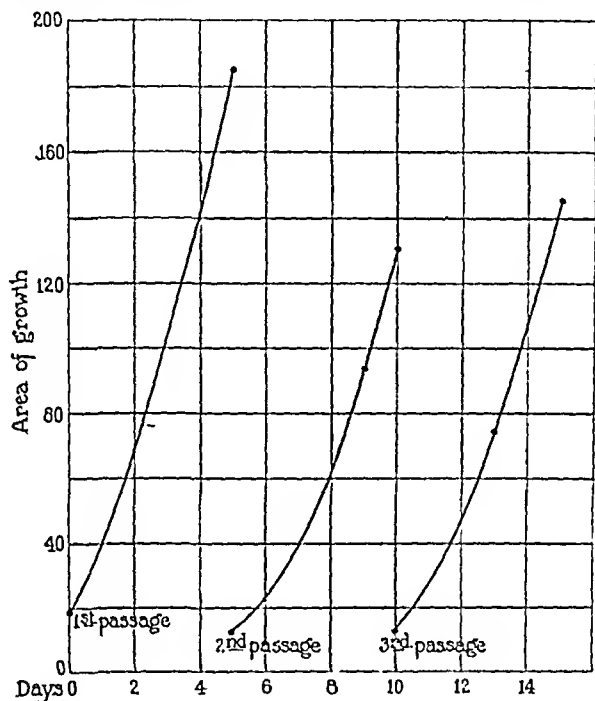
Text-Fig. 10. Experiments 4391-A, 4416-A and 4440-A. Growth of normal fibroblasts of the rat in the alcohol-soluble fraction of Cohn's extract of liver. After the fourth passage, the rate of growth decreased markedly. Concentration of nitrogen, .0075 per cent.

It is evident that protein hydrolytic products including proteoses are more toxic to normal than to sarcoma cells. Since sarcoma cells, as shown by Warburg,[21] and observed also by us in pure cultures of sarcomatous fibroblasts,[22] possess a great glycolytic activity as compared with normal fibroblasts, the medium surrounding them is

[21] Warburg, O., Biochem. Z., 1923, cxlii, 317; Warburg, O., Posener, K., and Negelein, E., Biochem. Z., 1924, clii, 309.

[22] Carrel, A., and Ebeling, A. H., J. Exp. Med., 1928, xlviii, 105.

always acid. Its pH is approximately 6.0, while that of the medium surrounding normal fibroblasts is about 7.0. Between these concentrations of hydrogen ion the enzyme activity varies greatly. It seems quite plausible, therefore, that sarcoma cells synthetize protoplasm from peptides and peptones more readily than normal cells. As shown by Wasteneys and Borsook,[23] the synthesis of protein from proteolytic products of albumin by trypsin takes place readily at a hydrogen ion concentration varying from 5.5 to 6.5. It may equally well be that sarcoma cells have evolved enzymes which function somewhat differently from those of normal cells. Whatever the explanation of this difference, it is evident that sarcoma cells are able to proliferate rapidly and indefinitely on the products of enzymatic hydrolysis of proteins. This fact sheds further light on the mechanism of the growth of the tumor within the body. Any process bringing about protein digestion, such as takes place when dead or injured tissue is autolyzed by the enzymes set free or by macrophages, would supply sarcomatous fibroblasts with nutrient material.

CONCLUSIONS.

1. Both normal and sarcomatous fibroblasts of the rat utilize many different fragments of the protein molecule for their growth *in vitro*. Alpha and beta proteoses have approximately equal growth-promoting power.

2. A mixture of peptones, peptides, and amino acids, containing a negligible quantity of proteose, produces a temporary proliferation of normal fibroblasts, and an unlimited multiplication of sarcomatous fibroblasts, provided these substances are derived from liver which contains products of unknown nature that complete the nutritive effect of the protein degradation products.

3. Amino acids contribute to the nutrition of the cells, but are unable without the addition of peptides or polypeptides to support their life.

4. The proteolytic products are more toxic to normal than to sarcomatous fibroblasts. The hypothesis is suggested that the greater acidity produced by the large glycolysis of the sarcomatous cells may account for this difference through altering the speed of action of protein synthetizing enzymes.

[23] Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1925, lxiii, 575.
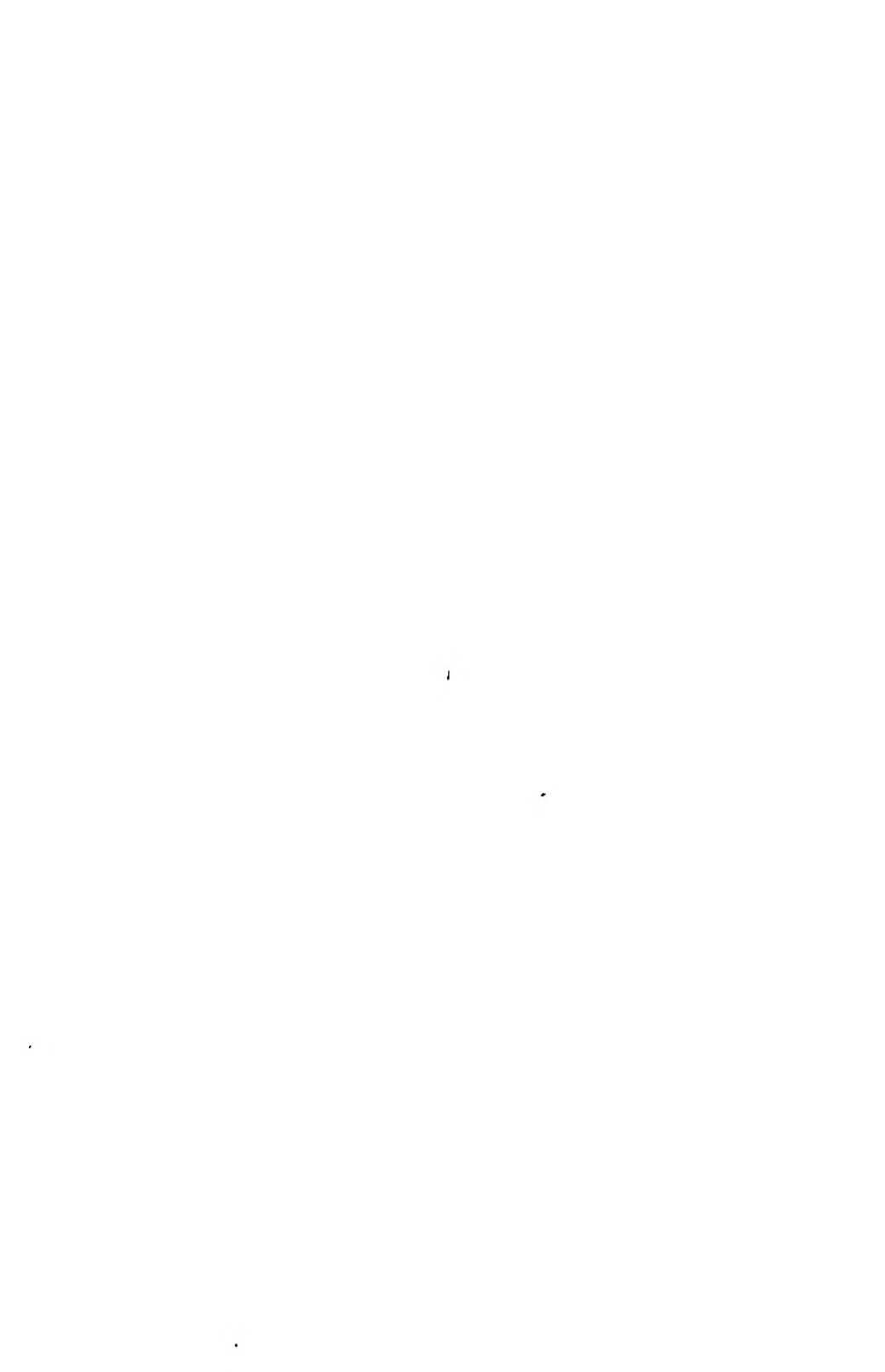
BIOMETRY OF CALCIUM, INORGANIC PHOSPHORUS, CHOLESTEROL, AND LIPOID PHOSPHORUS IN THE BLOOD OF RABBITS.

I. Normal Animals from Recently Acquired Stock.

By ALVIN R. HARNES, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The study presented by Brown (1) of calcium and inorganic phosphorus in the blood serum of normal rabbits showed certain definite results with respect to variation of these two elements over a given period of time. It seemed desirable to continue and extend these observations with a view to determining whether the variations that occurred followed a similar course from year to year and whether the changes in calcium and phosphorus were associated with corresponding variations in other constituents of the blood. In addition to calcium and inorganic phosphorus in the blood serum, two constituents of the whole blood were added, namely, cholesterol and lipoid phosphorus. The results obtained from these analyses of the blood are presented to show the degree of variation of each of these 4 constituents and a mathematical correlation of these variations. The experiments may be divided into two general groups: first, Group 1 to include all normal animals living under various environmental conditions; second, Group 2 to include diseased animals which have been inoculated either with *Treponema pallidum* or a malignant tumor. Each group under these two general classes will be presented separately.

Material and Methods.

The present discussion will be limited to those animals recently removed from outside living conditions, so that the results presented are, as nearly as possible, representative of an animal living out in the open.

From October 20, 1927, to May 15, 1928, determinations of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus were made on 80 animals consisting of 8 groups of 10 each. The monthly determinations were made on a separate group of animals recently received by the laboratory from the breeding farms. The approximate age of the animals in each monthly group was between 6 and 8 months. In each instance, 10 animals were used, the mean values of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus for the 10 animals determined, with their respective probable errors, the standard deviation from the means and the coefficients of variation. The curves for calcium, inorganic phosphorus, cholesterol and lipoid phosphorus in Fig. 1 were all plotted with the respective mean values given in mg. per 100 cc. Thus each monthly determination represents mean values for 10 animals, making a total of 80 animals examined over the period of 8 months. Except for the group of 10 animals examined on May 15, none of the animals were in the laboratory longer than 48 hours before the blood was examined, and in the last group the examination was made 72 hours after the animals were received in the laboratory.

On the day preceding examination, animals were fed as usual in the afternoon and were bled early the following morning before they had received additional food. The blood used for all determinations was drawn from the marginal ear vein of the rabbit. For cholesterol and lipoid phosphorus, 3 cc. of blood were drawn into a small test-tube into which had previously been placed 0.1 gm. sodium oxalate, the tube being constantly agitated until the total amount required was collected. Immediately after the blood for cholesterol and lipoid phosphorus was collected, 5 cc. of blood were drawn from the same opening into a graduated centrifuge tube and permitted to clot at room temperature. As soon as the clot was formed, it was separated from the side of the tube and centrifuged at 1400 revolutions per minute for 15 minutes. The lapse of time between the bleeding and the beginning of chemical analysis was never more than 2 hours, thus reducing to a minimum any error which might be due to the specimen of blood remaining too long at room temperature.

For the calcium and inorganic phosphorus the serum was measured with a 1 cc. tuberculin syringe graduated to 0.01 cc. and fitted with a 20 gauge needle. The whole blood for cholesterol and lipoid phosphorus was measured in like manner. Determinations of calcium and inorganic phosphorus were made on the blood serum according to the methods devised by Kramer and Tisdall (2) and Tisdall (3) respectively.

Cholesterol was determined by a slight modification of the method devised by Autenrieth (4), the modification consisting in the manner in which the cholesterol and lipoid phosphorus were extracted from whole blood; sodium oxalate in crystalline form being used as a non-coagulant. 1 cc. of whole blood was spread over a strip of fat-free filter paper 1½ by 7 inches. Two strips were used for each sample of blood, one for the determination of cholesterol and the other for the determination of lipoid phosphorus. These strips were then dried in an electric oven at 50°C.

The lipoid phosphorus was determined by the method described in a previous paper (5). The extraction of cholesterol with CHCl₃ was carried out in the same manner as described in the method for the determination of lipoid phosphorus. After the extraction with CHCl₃ was complete, the sugar tubes containing the cholesterol extract were placed in an electric oven at 100°C. until all the CHCl₃ had evaporated. The residue was taken up with small quantities of CHCl₃, transferred to a 15 cc. capacity Pyrex centrifuge tube and made up to 5 cc. 2 cc. acetic anhydride and 0.1 cc. H_2SO_4 were added and the color developed after standing 10 minutes was read against 5 cc. of a standard solution of cholesterol in a colorimeter. The standard solution of cholesterol was prepared by dissolving 20 mg. of cholesterol in 100 cc. of CHCl₃. 5 cc. of the standard solution are equal to 1 mg. cholesterol. In performing a series of duplicate analyses on the same sample of blood for the purpose of checking results and determining the degree of experimental error, the following average results were obtained: calcium 2.3 per cent, phosphorus 1.7 per cent, cholesterol 3.3 per cent and lipoid phosphorus 4.8 per cent variation in 100 cc. of sample.

RESULTS.

The results of analyses made in this experiment are presented in Text-figs. 1 to 7 and Tables I to VII. In all calculations, figures and tables, the lipoid phosphorus is calculated as lecithin, these calculations being made on the basis of results obtained by Levene (6) in which the phosphorus in lecithin is calculated as being approximately 4 per cent.

DISCUSSION AND CONCLUSIONS.

The following experiment was performed to demonstrate the variation of calcium in the same sample of blood serum when kept under two conditions of temperature.

30 cc. of blood were drawn by means of a hypodermic needle and syringe from the heart of a rabbit. The blood was divided into two portions of 15 cc. each and duplicate analyses for calcium were made on the serum immediately after centrifuging. The remaining portion of one sample was then corked and placed in the open laboratory; the other sample was corked and placed in the refrigerator at 4°C. The following are the results obtained:

Time after blood was drawn	Room temperature	Refrigerator
hrs.	mg. per 100 cc.	mg. per 100 cc.
0.5	13.4	13.6
1.5	13.0	13.6
24.0	12.6	13.6
52.0	13.2	14.2

TABLE I.

Values for Consecutive Monthly Determinations.

Date	Calcium			Inorganic phosphorus			Cholesterol			Lecithin		
	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
1927	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*	*mg. per 100 cc.*	*mg.*	*per cent*
Oct. 20	18.5 ±0.394	3.59	19.41	6.045 ±0.173	0.811	13.42	51.1 ±1.188	5.57	10.90	94.8 ± 1.397	6.55	6.91
Nov. 17	16.1 ±0.158	0.740	4.59	5.230 ±0.190	0.890	17.01	64.5 ±1.89	8.89	13.78	135.8 ± 3.10	14.55	10.71
Dec. 15	14.7 ±0.156	0.733	4.98	6.811 ±0.220	1.06	15.69	68.7 ±2.94	13.79	20.08	100.3 ± 3.74	17.54	17.48
1928												
Jan. 12	14.5 ±0.100	0.469	3.23	6.109 ±0.190	0.890	14.58	73.3 ±1.342	6.29	8.58	125.1 ± 1.66	7.79	5.82
Feb. 21	15.8 ±0.180	0.845	5.38	4.960 ±0.204	0.958	19.31	71.2 ±1.85	8.69	12.21	168.3 ±10.18	47.75	23.85
Mar. 13	15.1 ±0.144	0.677	4.48	5.431 ±0.271	1.27	23.38	71.7 ±1.99	9.34	13.02	152.2 ± 2.75	12.88	8.46
Apr. 19	15.1 ±0.168	0.786	5.21	6.820 ±0.205	0.962	14.11	66.5 ±1.45	6.80	10.23	99.4 ± 2.50	13.83	13.91
May 15	14.5 ±0.162	0.760	5.24	5.245 ±0.167	0.785	14.97	67.6 ±2.293	10.75	15.90	118.6 ± 2.664	12.49	10.53

From the results of this experiment it may be seen that in the serum of the blood held at room temperature the calcium decreased 0.8 mg. per 100 cc. in 24 hours, while that held in the refrigerator was practically constant. 52 hours after the blood was drawn, both samples showed an increase in calcium of 0.6 mg. per 100 cc. from their preceding values. The sample held at room temperature was found

TABLE II.

Distribution of calcium		Distribution of calcium	
mg. per 100 cc.		*mg. per 100 cc.*	
12.5–13.4	2	19.5–20.4	0
13.5–14.4	23	20.5–21.4	2
14.5–15.4	25	21.5–22.4	1
15.5–16.4	18	22.5–23.4	0
16.5–17.4	4	23.5–24.4	1
17.5–18.4	2		
18.5–19.4	2	Observations........80	

TABLE III.

Distribution of inorganic phosphorus	
mg. per 100 cc.	
3.00–3.99	3
4.00–4.99	15
5.00–5.99	24
6.00–6.99	26
7.00–7.99	9
8.00–8.99	3
Observations....80	

TABLE IV.

Distribution of cholesterol	
mg. per 100 cc.	
30–39.9	1
40–49.9	3
50–59.9	19
60–69.9	26
70–79.9	22
80–89.9	6
90–99.9	3
Observations..80	

TABLE V.

Distribution of lecithin	
mg. per 100 cc.	
70– 99.9	20
100–129.9	31
130–159.9	21
160–189.9	5
190–219.9	1
220–249.9	2
Observations....80	

to contain 1.0 mg. per 100 cc. less calcium than the sample held at 4°C. at the end of 52 hours.

Variation.—Fig. 1 is a graphic presentation of the results listed in Table I. An analysis of the results obtained for calcium shows the highest value of 18.5 ±0.36 mg. per 100 cc. of blood serum occurring in October. During November, December and January there occurred a gradual decrease in calcium from the above value until on

TABLE VI.

Coefficients of Correlation for Consecutive Monthly Determinations.

Date	rP. Ca	P.E.	rP. Chol.	P.E.	rP. Lec.	P.E.	rCa. Chol.	P.E.	rCa. Lec.	P.E.	rChol. Lec.	P.E.
1927												
Oct. 20	−0.444	±0.171	−0.133	±0.210	+0.031	±0.213	−0.295	±0.194	+0.205	±0.204	+0.341	±0.188
Nov. 17	−0.100	±0.211	+0.021	±0.213	−0.175	±0.207	+0.370	±0.184	+0.443	±0.171	+0.202	±0.205
Dec. 15	−0.438	±0.172	+0.519	±0.156	+0.029	±0.213	−0.170	±0.207	+0.162	±0.208	+0.002	±0.213
1928												
Jan. 12	−0.111	±0.211	+0.076	±0.212	+0.104	±0.211	+0.812	±0.073	−0.175	±0.207	−0.127	±0.210
Feb. 21	−0.709	±0.106	−0.404	±0.178	−0.201	±0.205	+0.115	±0.210	+0.250	±0.200	+0.112	±0.211
Mar. 13	−0.520	±0.156	+0.049	±0.213	+0.458	±0.169	−0.291	±0.195	−0.134	±0.209	+0.175	±0.207
Apr. 19	−0.423	±0.175	−0.220	±0.203	−0.118	±0.210	−0.220	±0.203	+0.495	±0.161	+0.099	±0.211
May 15	+0.476	±0.165	+0.404	±0.178	+0.302	±0.194	+0.495	±0.161	−0.045	±0.213	−0.118	±0.211

TABLE VII.

Section (a). Coefficients of Correlation of Group Means.

rP. Ca	P.E.	rP. Chol.	P.E.	rP. Lec.	P.E.	rCa. Chol.	P.E.	rCa. Lec.	P.E.	rChol. Lec.	P.E.
−0.094	±0.236	−0.137	±0.234	−0.794	±0.088	−0.887	±0.051	−0.235	±0.225	+0.560	±0.164

Section (b). Coefficients of Correlation with 80 Individual Values.

rP. Ca	P.E.	rP. Chol.	P.E.	rP. Lec.	P.E.	rCa. Chol.	P.E.	rCa. Lec.	P.E.	rChol. Lec.	P.E.
−0.146	±0.073	+0.002	±0.075	−0.347	±0.066	−0.340	±0.067	−0.088	±0.075	+0.285	±0.069

January 12, the amount of calcium was found to be 14.5 ±0.10. From January 12 to February 21, the calcium increased from 14.5 ±0.10 to 15.8 ±0.18 mg. per 100 cc. of blood serum. In February and through March, April and May the calcium gradually decreased in amount until on May 15 the mean value for the 10 animals was found to be 14.5 ±0.16. Thus the two extreme mean values for calcium during the 8 months of this experiment were found to be 18.5
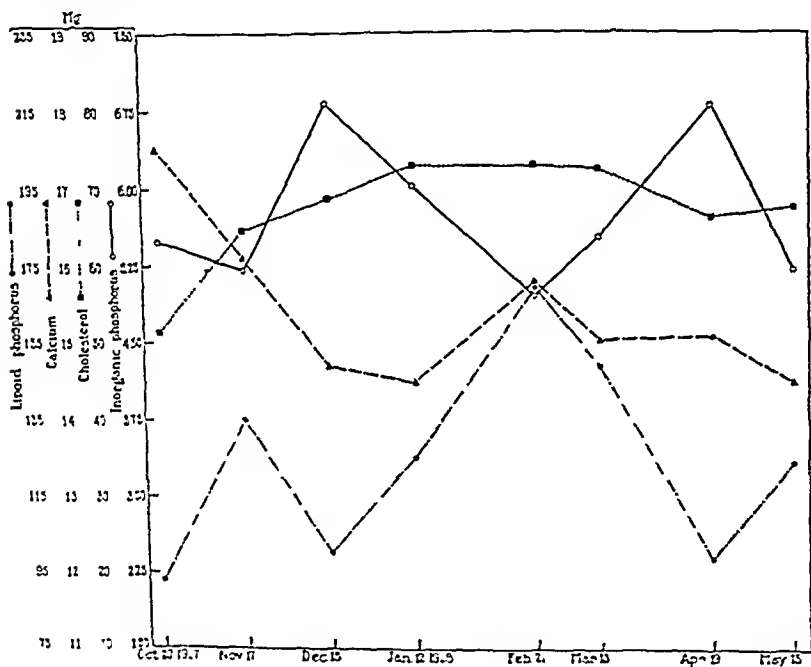
TEXT-FIG. 1.

±0.36 and 14.5 ±0.10 mg. per 100 cc. of blood serum. That this variation is of mathematic significance is demonstrated by the small probable error when compared with the difference between the two extremes. The results obtained are due to actual variations occurring in the blood serum and are not due to error of technique or chance sampling.

In actual mg. of the element, the variation of inorganic phosphorus

TEXT-FIG. 2.

Distribution of calcium

Mg. per 100 cc. serum

TEXT-FIG. 3.

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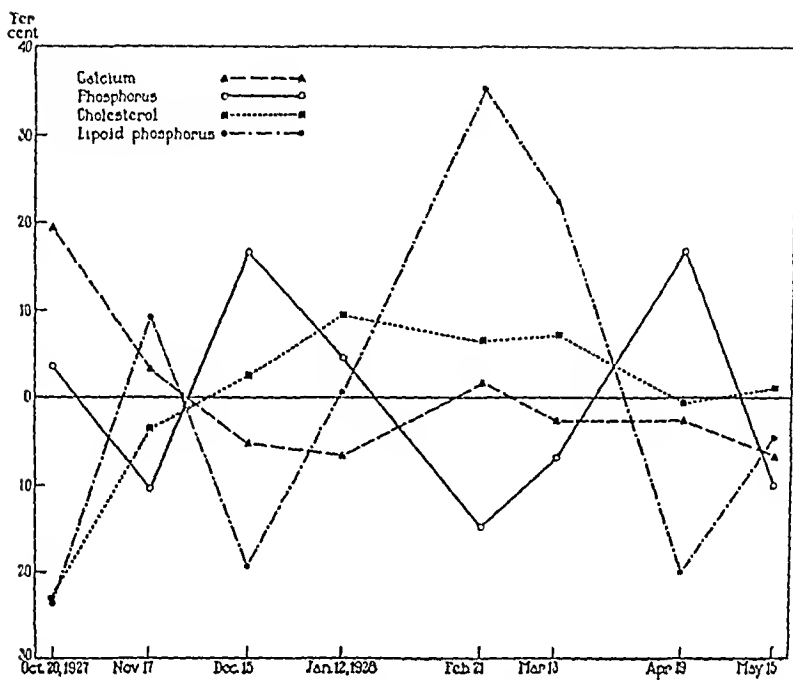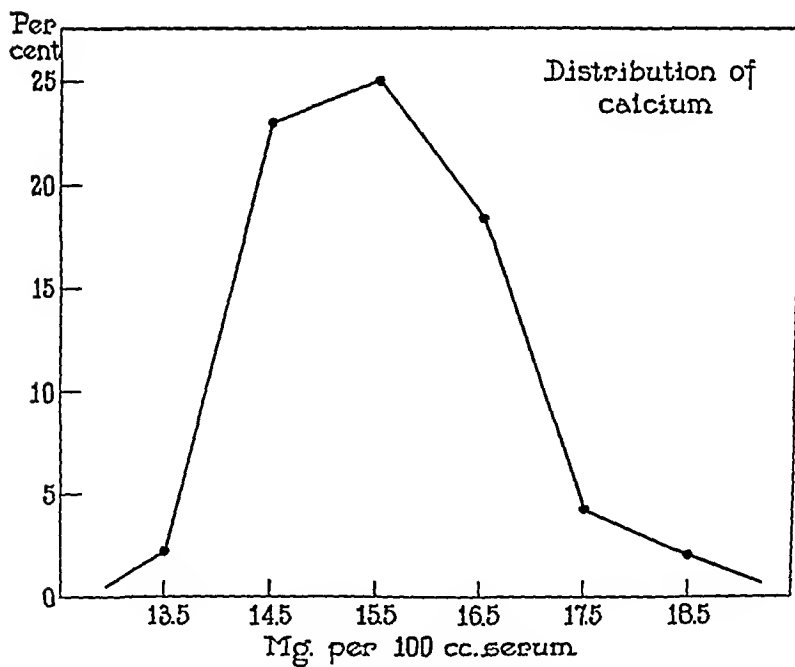
in the blood serum was found to be less than any one of the other 3 constituents. The frequency of fluctuation of phosphorus, however, was found to be greater than any one of the other constituents. When the mean value of 5.813 ±0.088 for phosphorus is considered, the variation of ±1.00 mg. occurring throughout the experiment is of high
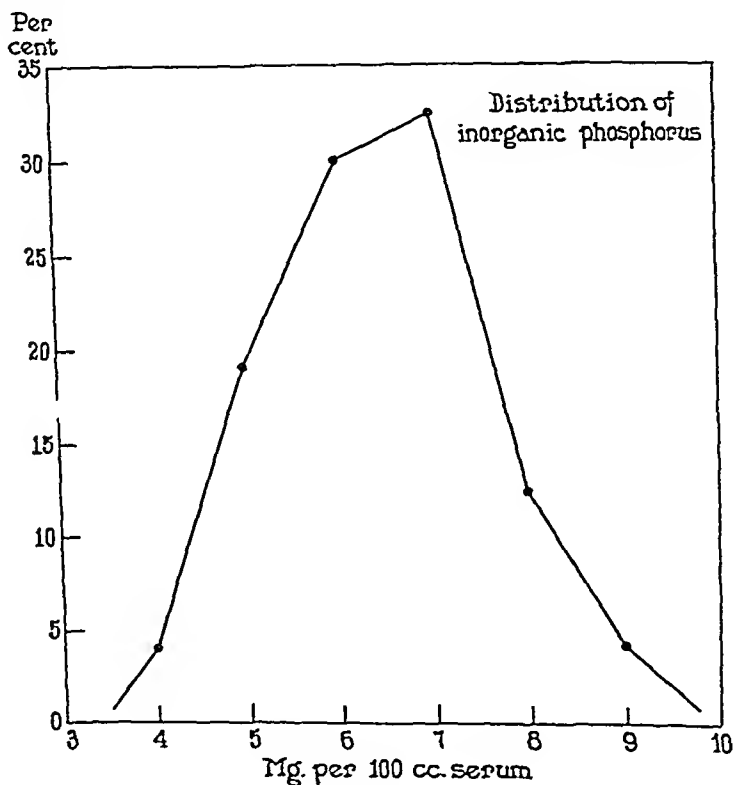
TEXT-FIG. 4.

magnitude and can in no way be related to variation due to chance. From October to November, the inorganic phosphorus showed a decrease from 6.045 ±0.17 to 5.230 ±0.19 mg. per 100 cc. of blood serum. During December there was a marked increase reaching 6.811 ±0.22 on December 15. Through January and February the inorganic phosphorus decreased in amount until on February 21 the

mean value was found to be 4.960 ±0.20 mg. per 100 cc. of blood serum, this being the lowest value in the series of 8 monthly examinations. In March and April the inorganic phosphorus exhibited another marked increase similar to the increase first observed during November and December, reaching 6.820 ±0.20 mg. per 100 cc. of
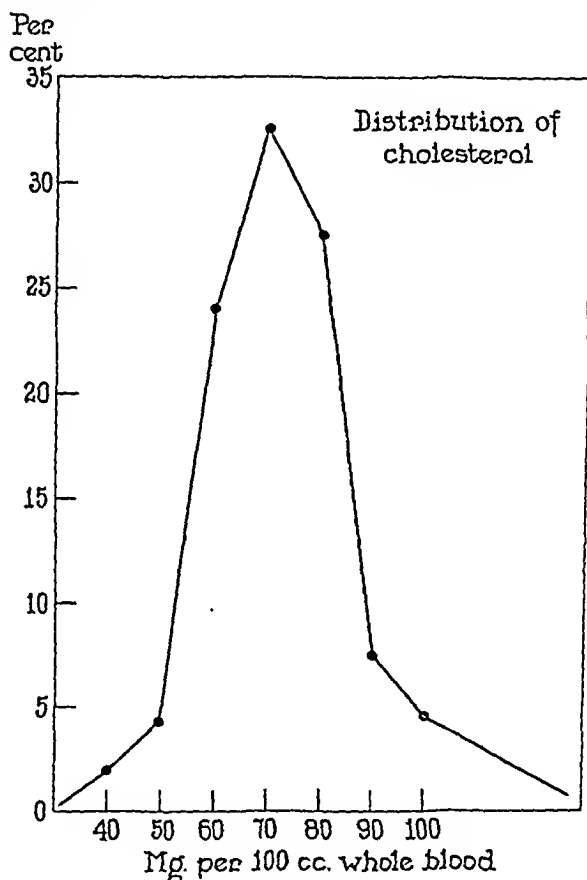
TEXT-FIG. 5.

blood serum on April 19. From this high value the trend was downward until on May 15 the amount of inorganic phosphorus was 5.245 ±0.16 mg. per 100 cc. of blood serum.

The stability of cholesterol was probably greater than any of the other 3 constituents. Beginning on October 20 with a mean value of 51.1 ±1.1 mg. per 100 cc. of whole blood, the cholesterol gradually

increased in amounts throughout November, December and on January 12 reached 73.3 ±1.3 mg. per 100 cc. of whole blood.   During January, February and March the amounts of cholesterol found in the blood maintained a practically constant value.   During March and
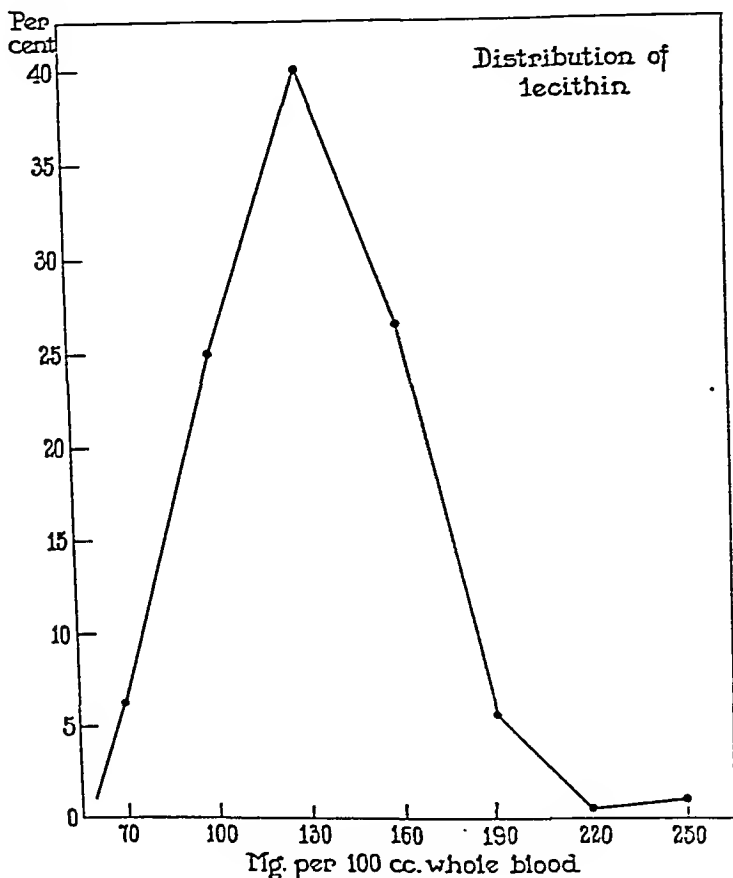
TEXT-FIG. 6.

April the cholesterol showed a slight decrease until on April 19 the mean value was found to be 66.5 ±1.4 mg. per 100 cc. of whole blood. The regularity of the curve for cholesterol suggests that this constituent of the whole blood shows the least degree of variation throughout the experiment.

Lipoid phosphorus, which in Fig. 1 is calculated and presented as lecithin, began with a value of 94.8 ±1.3 mg. per 100 cc. of whole blood. An increase occurred during October and November until on November 17 the lecithin was calculated to be 135 ±3.1 mg. per 100 cc.   On December 15 the mean value for this group of animals was found to be 100.3 ±3.7, a marked decrease in amount of lecithin as compared
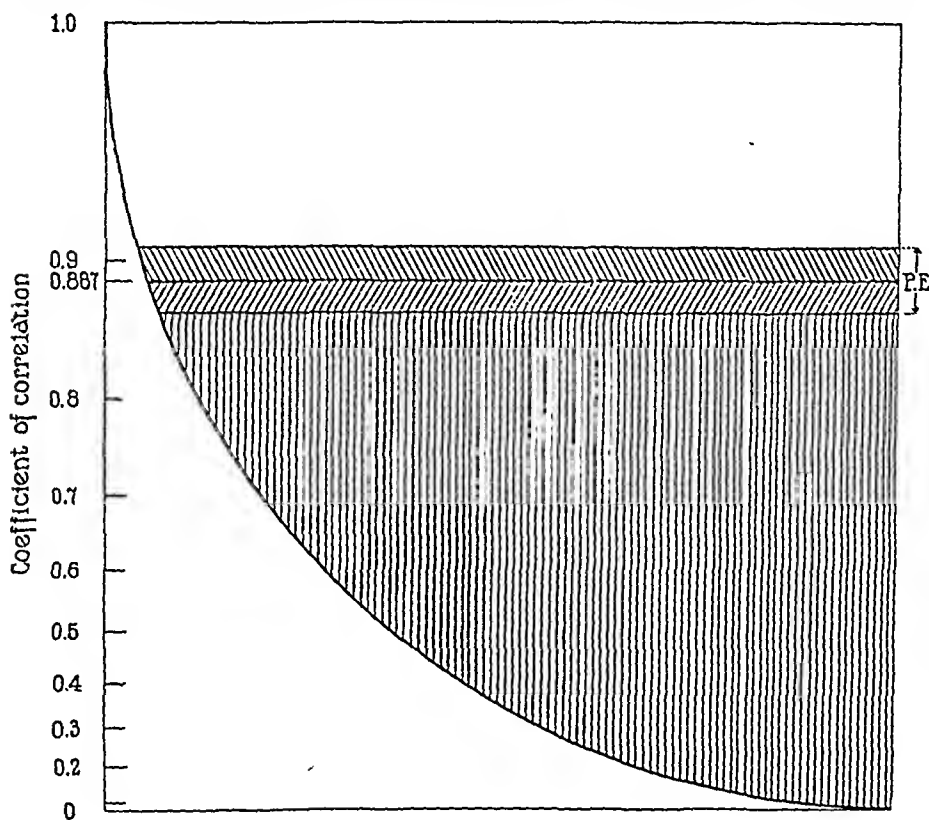
TEXT-FIG. 7.

with the previous monthly groups examined.   During December and January there occurred a pronounced increase in lecithin until on February 21, 168.3 ±10.1 mg. per 100 cc. of whole blood was found to be present.   The consequent decrease in lecithin during March and April reached its lowest level on April 19 at which time the lecithin in the whole blood was found to be 99.4 ±2.5 mg. per 100 cc.   A slight increase occurred during the latter part of April and on May 15

the lecithin was found to be 118.6 ±1.6 mg. per 100 cc. of whole blood. Lecithin, when compared to the other 3 constituents, exhibited the greatest degree of variation. The difference between the two extreme values was 73.5 mg. or a fluctuation about its mean value of −29.7 and +33.8 mg. A comparison of the general average of calcium obtained in the present series of animals with the value for last year shows the following results.

	Results of Brown	Author's results
Calcium...	15.4 ±0.09	15.5 ±0.14
Phosphorus.....................................	5.40 ±0.06	5.81 ±0.08

The trend of the per cent variation for each of the 4 constituents from their respective averages is presented in Fig. 2. The similarity between Figs. 1 and 2 is due to the fact that the divisions in mg. per 100 cc. in Fig. 1 were so calculated for calcium, inorganic phosphorus, cholesterol and lecithin that the variation of one was recorded in the same degree as any one of the other 3 constituents. The greatest extreme per cent variation occurred with lecithin, the values for this constituent of the whole blood ranging from +35 per cent to −20 per cent of the mean value 124.5 ±2.4. Inorganic phosphorus which in mg. per 100 cc. of serum had the least value gave a per cent variation from +18 to −15 per cent of the mean value 5.81 ±0.08. The per cent variation for calcium was from +20 to −5 per cent of the mean value 15.5 ±0.14. Cholesterol varied from +10 to −22 per cent of the mean value 66.8 ±0.84. While it is evident that these variations follow a seasonal trend, this subject will be discussed in a subsequent paper.

Averaging the 80 determinations made for each of the 4 constituents, the mean value for calcium was found to be 15.5 ±0.14; inorganic phosphorus 5.813 ±0.088 mg. per 100 cc. of blood serum; for cholesterol 66.8 ±0.846 and lecithin 124.5 ±2.47 mg. per 100 cc. of whole blood. Tables II, III, IV and V illustrated respectively with Figs. 3, 4, 5 and 6 refer to the distribution of calcium, inorganic phosphorus, cholesterol and lecithin about their respective mean values.

Correlation.—The trends of calcium, inorganic phosphorus, chol-

esterol and lecithin illustrated in Fig. 1 when taken for the entire 8 months, exhibit certain striking facts indicating possible relationships which may exist between these blood constituents. For example, the curve for inorganic phosphorus is almost a mirror image of the curve for lecithin; calcium and inorganic phosphorus show a similar relationship only to a less degree. With these facts in mind, Pearson's (7) formula for the measurement of the coefficient of correlation was applied to the 6 possible combinations of these 4 elements, that is, phosphorus $\times$ calcium, phosphorus $\times$ cholesterol, phosphorus $\times$ lecithin, calcium $\times$ cholesterol, calcium $\times$ lecithin and cholesterol $\times$ lecithin. Table VI gives the results with the probable errors obtained by calculating the correlation existing in each group of monthly determinations of 10 animals; Table VII gives the results with probable error obtained (a) with the mean values obtained for each monthly determination and (b) with the 80 animals used as one group. Section (a), therefore, represents the coefficient of correlation for the trend of the curves presented in Fig. 1.

Each of the results reported in Tables VI and VII, Sections (a) and (b), was calculated by the formula $r = \dfrac{\Sigma\, XY}{N \cdot \sigma X \cdot \sigma Y}$ in which

> r = coefficient of correlation for each of the 6 possible combinations.
>
> N = number of cases from which the mean value was derived.
>
> $\Sigma X\, Y$ = the algebraic sum of the products of the deviations from the respective means for each series.
>
> σ = standard deviations of the X and Y distributions respectively.

In order to obtain the value for $\Sigma X\, Y$, the mean value of each of the 4 constituents is computed, that is, mean value for calcium, phosphorus, cholesterol and lecithin. The difference between this mean value and each single result is next calculated, maintaining, of course, the proper algebraic sign. Next the products of these differences for each combination, taking 2 constituents at a time are computed, that is, phosphorus $\times$ calcium, phosphorus $\times$ cholesterol, etc., maintaining throughout the proper sign. The Σ is then obtained by computing the algebraic sum of these product moments.

It is, therefore, quite evident from the above formula that unless there exists a difference between each separate determination and the mean value of the respective series, there can be no coefficient of correlation. For example, if 10 animals gave a mean value for calcium of 14.6 mg. per 100 cc. of serum and *all the animals in the group had*

the same value as the mean, the coefficient of correlation for that series with any one of the other constituents would be zero, due to the fact that the product moments of $X\ Y$ and the resulting $\Sigma\ X\ Y$ would be zero. In the results reported in Table VI, whatever factor or factors caused the values either to increase or to decrease, exerted its influence more or less equally on all the animals in the group so that the product moments and the resulting $\Sigma\ X\ Y$ was comparatively small. This fact is demonstrated by the results reported in Table VI in which the coefficient of correlation was calculated for each group of 10 animals.

In order to measure mathematically the trend of relationship of these 4 blood constituents throughout the period of 8 months, the coefficient of correlation was calculated by using the group means as presented in Fig. 1 and Table I. The results obtained from this calculation are presented in Table VII, Section (a) with their respective probable errors ($P.E.$). Of these 6 values, 3 stand out as being of mathematical significance, namely,

$$r \text{ phosphorus} \times \text{lecithin} = -0.794 \pm 0.088$$
$$r \text{ calcium} \times \text{cholesterol} = -0.887 \pm 0.051$$
$$r \text{ cholesterol} \times \text{lecithin} = +0.560 \pm 0.164$$

In each of the above results the coefficient of correlation is 3 times or more the probable error. If the coefficient is 3 times the probable error, the odds against the occurrence of a difference as great or greater than this in proportion to its probable error are 32 to 1, if chance alone were operative in the determination of the event. If we take the values of the coefficient of correlation for r phosphorus $\times$ lecithin and r calcium $\times$ cholesterol, the probabilities due to chance are even less than that calculated for r cholesterol $\times$ lecithin. Of the remaining 3 correlations, attention is called to the relation existing between calcium and phosphorus. While the sign of the coefficient in all cases except one is negative, the calculated probable error in this series does not permit a conclusive statement being made concerning this relationship.

With these values for the coefficient of correlation, the question arises as to what degree one may predict the trend of either constituent if one or the other is known. Fig. 7 illustrates the factor in per cent

above chance for the value obtained from r calcium $\times$ cholesterol. The shaded area, which is derived by drawing a line parallel to the abscissa through the value for the coefficient of correlation, represents a sector of a quadrant of a circle. The per cent of the total area of this sector as compared with the area of the whole quadrant is the factor above chance that calcium and cholesterol will continue in their stated relationship at the time of the next determination. With a coefficient of correlation for calcium $\times$ cholesterol of -0.887 ± 0.051, the probability in favor of the present trend continuing is 50.7 per cent above chance, or a 2 to 1 ratio.

The general average obtained for the 80 animals examined gave a value for inorganic phosphorus of 5.81 ± 0.08 mg. and 15.5 ± 0.14 mg. for calcium per 100 cc. of serum. For cholesterol the mean value was found to be 66.8 ± 0.84 mg. and for lecithin 124.5 mg. per 100 cc. of whole blood.

The coefficients of correlation calculated for the trend of these 4 blood constituents were found to be

r phosphorus $\times$ lecithin	=	-0.794 ± 0.08
r calcium $\times$ cholesterol	=	-0.887 ± 0.05
r cholesterol $\times$ lecithin	=	$+0.560 \pm 0.16$
r phosphorus $\times$ calcium	=	-0.094 ± 0.23
r phosphorus $\times$ cholesterol	=	-0.137 ± 0.23
r calcium $\times$ lecithin	=	-0.235 ± 0.22

SUMMARY.

Determinations of calcium, inorganic phosphorus, cholesterol and lipoid phosphorus were made on a series of animals recently received from the dealer for the purpose of determining the trends of these 4 blood constituents throughout the year with the degree of their respective variations and mathematical correlation. It was found that for the 80 animals examined, calcium varied from 14.5 ± 0.10 to 18.5 ± 0.39 mg. and inorganic phosphorus 4.960 ± 0.20 to 6.820 ± 0.20 mg. per 100 cc. of blood serum. Cholesterol varied from 51.1 ± 1.18 to 73.3 ± 1.34 mg. and lecithin from 94.8 ± 1.397 to 168.3 ± 10.18 mg. per 100 cc. of whole blood.

Of the 6 possible combinations in calculating the coefficient of correlation for the trend throughout the experiment, 3 stand out as of

mathematical significance, namely the following. Between inorganic phosphorus and lecithin the coefficient of correlation was found to be −0.794 ±0.088; between calcium and cholesterol −0.887 ±0.051 and between cholesterol and lecithin +0.560 ±0.164.

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INFLUENCE OF LIGHT ENVIRONMENT ON THE ORGANIC CONSTITUTION OF NORMAL RABBITS WITH ESPECIAL REFERENCE TO THE ACTION OF NEON LIGHT.

By WADE H. BROWN, M.D.

WITH THE COLLABORATION OF MARION HOWARD.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

From observations made on normal rabbits, it was found that nutrition and growth and the growth of hair may be influenced by living in an environment of neon light, in the dark, or in an environment of diffuse, filtered sunlight of varying intensity, and that the effects produced by these conditions depend to some extent upon the color of the animal (1, 2).

When the observations on the rabbits used for these experiments had been completed, the animals were killed and their organs weighed for the purpose of determining whether any difference in the organic constitution of the several groups of animals could be detected, and whether a relation could be established between physical and functional states presented by animals living under different environmental conditions. The results of this phase of the experiments will be reported in the present paper.

Material and Methods.

The report is based on an analysis of the organ weights of 3 groups of normal rabbits. Each of the groups contained 15 animals, 5 of which had been living in an environment of neon light, 5 in the dark, and 5 received diffuse, filtered sunlight of varying intensity. Group I was composed of white, Group II of black, and Group III of gray, brown, and black animals.

The animals of Groups I and II were placed in their respective environmental conditions October 22, 1926, and remained under these conditions until they were killed on May 18 and 19, 1927, or for a period of approximately 7 months. The observations on the animals of Group III began October 1, 1926. On February 8, 1927, the light and dark divisions of this group were interchanged, so that

animals which had been living in an environment of neon light for approximately 4 months were placed in the dark and *vice versa*. This condition was maintained until May 4, 1927, when the two groups were restored to their original environments. The animals were killed June 8 and 9, or 5 weeks after the second change was made.

The organs were weighed in accordance with methods which have been described in detail elsewhere (3). The mean values obtained for actual weights of organs and for the weights per kilo of net body weight (relative weight) (3) are recorded in Table I.

For comparative purposes certain standard values are also given in Table I. These were derived from an analysis of body and organ weights of 645 normal rabbits (4). The standard mean for the weight of each organ is the mean value obtained for the 645 animals and represents the weight of organs for rabbits with a mean gross body weight of 2262 gm. or a net body weight of 1841 gm. The figures given in the first column of Table I as standard values are the values obtained for animals with a net body weight corresponding with that of the light, dark, or control animals (5).

The values obtained in these experiments are first compared with corresponding standard values; and then, a direct comparison is made of the results thus obtained for light, dark, or control animals of a given or of a different group. In order to reduce the standard value to a single expression, a mean net body weight for the 3 groups of animals of a given class (light, dark, control) was obtained and the standard values given in Table I for gross body weight and for organs are those for animals with a net body weight corresponding with these means.

Differences between the values obtained in these experiments and the standard values for normal rabbits are recorded in Table I as percentage deviations— positive (+) or negative (−). In this way, a correction is made for differences in the weights of organs which might be due to differences in the weights of animals, and at the same time, the results for all classes of animals are reduced to a common basis of expression which permits of a direct comparison. The results obtained for the relative weights of organs, as recorded in Table I, are presented graphically in Text-figs. 1 to 9.

No standard values were available for the lungs, so that the results given in the tables and text-figures were obtained by direct comparison of light and dark animals with the controls of the same group.

The percentage differences between light, dark, and control animals are summarized in Table II. The figures given for light and dark animals represent differences between animals of these classes and the controls of the same group; the difference between the light and dark animals is then found by a comparison of these two values.

<div align="center">RESULTS.</div>

The results of the experiments are given in Tables I and II and Text-figs. 1 to 9.

TABLE I.

Actual and Relative Organ Weights in Gm. with Percentage Deviation from Standard Normal Values.

Organ	Stand-ard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Gronp III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Gross body weight		Standard mean [2262 gm.]					
Control	2796	2669	2740	2695	−4.54	−2.00	−3.61
Light	3044	2805	3110	2925	−7.85	+2.17	−3.91
Dark	2596	2455	2412	2715	−5.43	−7.09	−4.58
Gastrointestinal mass		Standard mean [419.9 gm.]					
Actual							
Control	432	417	408	410	−3.47	−5.56	−5.09
Light	504	404	455	445	−19.84	−9.72	−11.71
Dark	448	352	381	357	−21.43	−14.96	−20.31
Relative		Standard mean [232.2 gm.]					
Control	184	186	176	174	+1.09	−4.35	−5.43
Light	198	170	171	179	−14.14	−13.64	−9.60
Dark	208	169	189	153	−18.75	−9.14	−26.44
* Net body weight		Standard mean [1841 gm.]					
Control	2304	2296	2332	2285	− .35	+1.22	− .83
Light	2512	2401	2655	2480	−4.42	+5.69	−1.27
Dark	2164	2103	2031	2358	−2.82	−6.15	+8.97
Heart		Standard mean [5.28 gm.]					
Actual							
Control	6.86	6.50	7.03	6.02	−5.25	+2.48	−12.25
Light	6.51	6.84	7.17	6.81	+5.07	+10.14	+4.61
Dark	5.86	5.78	6.10	6.56	−1.37	+4.10	+11.95
Relative		Standard mean [2.87 gm.]					
Control	2.93	2.88	3.03	2.56	−1.71	+3.41	−12.63
Light	2.77	2.86	2.70	2.75	+3.25	−2.53	− .72
Dark	2.73	2.74	3.03	2.81	+ .37	+10.99	+2.93
Lungs—*cf.* Light and Dark with Control		Standard mean [ ]					
Actual							
Control		9.99	11.06	9.50			
Light		9.01	9.99	10.89	−9.81	−12.61	+13.91
Dark		8.97	8.26	10.75	−10.21	−28.03	+12.51
Relative		Standard mean [ ]					
Control		4.42	5.05	4.08			
Light		3.78	3.79	4.42	−14.48	−28.51	+7.69
Dark		4.27	3.97	4.57	−3.39	−24.43	+11.09

* The standard values for net body weight are the means for the 3 groups of animals of a given class.

TABLE I—*Continued.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Liver		Standard mean [85.8 gm.]					
Actual							
Control	86.9	87.0	76.6	69.6	+ .12	−11.9	−19.91
Light	90.5	74.0	83.0	78.6	−18.23	−8.29	−13.15
Dark	91.2	58.0	65.0	65.4	−36.40	−28.73	−28.29
Relative		Standard mean [47.4 gm.]					
Control	36.9	38.5	33.1	29.1	+4.34	−10.30	−21.14
Light	35.6	30.7	31.3	31.5	−13.76	−12.08	−11.52
Dark	42.5	27.5	32.1	28.0	−35.29	−24.47	−34.12
Kidneys		Standard mean [12.94 gm.]					
Actual							
Control	14.66	13.51	13.48	13.76	−7.84	−8.05	−6.14
Light	15.55	13.71	15.62	14.61	−11.83	+ .45	−6.05
Dark	13.79	11.89	11.87	12.13	−13.78	−13.92	−12.04
Relative		Standard mean [7.13 gm.]					
Control	6.25	5.98	5.80	5.86	−4.97	+2.48	+3.31
Light	6.56	5.68	5.94	5.91	−3.70	+3.20	+6.79
Dark	6.42	5.75	5.89	5.16	−2.23	+3.08	+11.05
Brain		Standard mean [9.17 gm.]					
Actual							
Control	9.66	9.18	9.90	9.98	−4.97	+2.48	+3.31
Light	10.01	9.64	10.33	10.69	−3.70	+3.20	+6.79
Dark	9.41	9.20	9.70	10.45	−2.23	+3.08	+11.05
Relative		Standard mean [5.08 gm.]					
Control	4.10	4.11	4.26	4.28	+ .24	+3.90	+4.39
Light	4.45	4.02	3.92	4.32	−9.66	−11.91	−2.92
Dark	4.38	4.38	4.79	4.46	± .00	+9.36	+1.83
Testicles		Standard mean [4.67 gm.]					
Actual							
Control	6.48	6.20	5.97	6.51	−3.32	−7.87	+ .46
Light	6.34	6.54	6.19	6.18	+3.16	−2.37	−2.52
Dark	5.48	5.56	4.93	5.35	+1.46	−10.04	−2.37
Relative		Standard mean [2.53 gm.]					
Control	2.76	2.77	2.56	2.82	+ .36	−7.25	+2.17
Light	2.77	2.74	2.33	2.51	−1.08	−15.88	−9.39
Dark	2.54	2.64	2.45	2.29	+3.94	−3.54	−9.84

TABLE I—*Continued*.

Organ	Stand-ard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	gm	gm	grs	per cent	per cent	per cent
Spleen		Standard mean [1 023 gm.]					
Actual							
Control	1 078	1 261	1 050	.976	+16.98	−2.60	−9 46
Light	1.334	1.071	.868	1 175	−19.64	−35.08	−11 92
Dark	1.140	.896	1.046	.908	−11 40	−8.25	−20 35
Relative		Standard mean [.546 gm.]					
Control	.458	.569	.454	.418	+24 24	− .87	−8 73
Light	.525	.449	.329	.475	−14.48	−37.33	−9.52
Dark	.530	.427	.538	.385	−19.43	+1.51	−27.36
Thymus		Standard mean [2.301 gm.]					
Actual							
Control	2.514	2.687	2.834	3.320	+6.96	+12.73	+22.06
Light	2.285	2.693	3 150	2 675	+17.86	+37.86	+17.07
Dark	2.526	2.737	2.503	2.878	+8.35	− .91	+13.94
Relative		Standard mean [1.264 gm.]					
Control	1.070	1.171	1.217	1.374	+9.44	+13.74	+28.41
Light	.897	1.123	1 223	1.081	+25.20	+36.34	+20.51
Dark	1.175	1.292	1.233	1.219	+9.96	+4.93	+3.75
Thyroid		Standard mean [2328 gm.]					
Actual							
Control	.294	.301	.432	.4756	+2 28	+46.79	+61.60
Light	.373	425	.881	.354	+13.85	+136.00	−5 17
Dark	.302	.316	.330	.3134	+4 60	+9.27	+3 74
Relative		Standard mean [.1265 gm.]					
Control	.1679	.1327	.185	.1881	−20.96	+10 19	+12 03
Light	.1579	.1756	.3345	.1413	+11 21	+111 84	−10 51
Dark	1406	.1493	.1603	.1322	+6 19	+14 01	−5 97
Parathyroids		Standard mean [01286 gm.]					
Actual							
Control	.0125	0135	.0146	0138	+8 00	+16 80	+10 40
Light	.0172	0162	0146	0148	−5 81	−15 12	−13 95
Dark	0139	.0129	0140	.0182	−7.19	+ .72	+30 91
Relative		Standard mean [00709 gm]					
Control	0053	00592	00626	00587	+11 70	+18 11	+10.76
Light	0075	00667	00553	00597	−11 07	−26 27	−20 40
Dark	0065	00616	00707	00781	−5 23	+8 77	+20 00

TABLE I—*Continued*.

Organ	Stand-ard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	gm.	gm.	gm.	per cent	per cent	per cent
Hypophysis				Standard mean [.0281 gm.]			
Actual							
Control	.031	.026	.031	.0284	−16.13	± .00	−8.39
Light	.0335	.0274	.0344	.0326	−18.21	+2.69	−2.69
Dark	.0303	.026	.028	.0262	−14.19	−7.59	−13.53
Relative				Standard mean [.0155 gm.]			
Control	.0133	.01137	.01338	.01243	−14.51	+ .60	−6.54
Light	.0139	.01137	.01312	.0131	−18.31	−5.61	−5.76
Dark	.01418	.01245	.01396	.0113	−12.20	−15.52	−19.75
Suprarenals				Standard mean [.3833 gm.]			
Actual							
Control	.559	.461	.664	.631	−17.53	+18.78	+12.88
Light	.583	.479	.640	.4752	−17.84	+9.78	−18.49
Dark	.427	.482	.529	.511	+12.88	+23.89	+19.67
Relative				Standard mean [.2082 gm.]			
Control	.238	.209	.290	.2705	−11.76	+21.85	+13.66
Light	.254	.1997	.2455	.1911	−21.38	−3.35	−24.46
Dark	.197	.2318	.2635	.2135	+17.67	+33.76	+8.38
Pineal				Standard mean [.01577 gm.]			
Actual							
Control	.0166	.016	.0174	.016	−3.61	+4.82	−3.61
Light	.0189	.017	.017	.0132	−10.05	−10.05	−30.16
Dark	.0164	.0156	.014	.0182	−4.88	−14.63	+10.98
Relative				Standard mean [.00871 gm.]			
Control	.0071	.00602	.00749	.00706	−15.21	+5.49	− .56
Light	.0074	.00665	.00618	.00534	−10.14	−16.49	−27.84
Dark	.0077	.00824	.00694	.00775	+7.01	−9.87	+ .65
Axillary lymph nodes				Standard mean [.1697 gm.]			
Actual							
Control	.160	.133	.112	.0976	−16.88	−30.00	−39.00
Light	.129	.157	.145	.1086	+20.16	+12.40	−15.00
Dark	.156	.148	.132	.0850	−5.13	−15.38	−45.51
Relative				Standard mean [.09368 gm.]			
Control	.0679	.0594	.0486	.0425	−12.52	−28.42	−37.41
Light	.0511	.0655	.0547	.0438	+28.18	+7.05	−14.29
Dark	.0728	.0701	.0658	.0350	−3.71	−9.62	−51.92

TABLE I—*Concluded.*

Organ	Standard value	Weight			Deviation		
		Group I	Group II	Group III	Group I	Group II	Group III
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
Popliteal lymph nodes		Standard mean [.2557 gm.]					
Actual							
Control	.251	.220	.207	.214	−12.36	−17.53	−14.74
Light	.223	.213	.223	.243	−4.48	± .00	+9.06
Dark	.244	.194	.203	.188	−20.33	−16.89	−23.03
Relative		Standard mean [.14066 gm.]					
Control	.1068	.0974	.0894	.0910	−8.80	−16.29	−14.81
Light	.0878	.0884	.0847	.0981	+ .68	−3.53	+11.73
Dark	.1135	.0952	.1021	.0792	−16.30	−10.04	−30.26
Mesenteric lymph nodes		Standard mean [3.46 gm.]					
Actual							
Control	4.18	3.42	2.14	2.49	−18.18	−48.80	−40.43
Light	4.42	2.81	2.30	2.58	−36.42	−47.96	−41.66
Dark	3.60	2.30	2.08	2.28	−36.11	−42.22	−36.67
Relative		Standard mean [1.885 gm.]					
Control	1.69	1.508	.917	1.09	−10.77	−45.74	−35.50
Light	1.74	1.187	.850	1.04	−31.78	−51.15	−40.23
Dark	1.68	1.090	1.020	.97	−35.12	−33.33	−42.26
Deep cervical lymph nodes		Standard mean [.1563 gm.]					
Actual							
Control	.178	.144	.214	.206	−19.10	+20.22	+15.73
Light	.166	.132	.217	.135	−20.48	+30.72	−18.67
Dark	.175	.146	.096	.208	−14.86	−45.03	+18.63
Relative		Standard mean [.0847 gm.]					
Control	.0756	.0664	.0926	.0901	−12.17	+22.49	+19.18
Light	.0656	.0564	.0850	.0542	−14.02	+29.57	−17.38
Dark	.0817	.0703	.0473	.0851	−13.97	−42.11	+4.16

TABLE II.

Difference in Per Cent between Control, Light, and Dark Divisions of the Same and of Different Groups.

Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Gross body weight						
Light	−3.31	+4.17	− .30			
Dark	+ .89	−5.09	− .97		.	
Difference	4.20	9.26	.67			
Net body weight						
Light	−4.07	+4.47	− .44			
Dark	−2.47	−4.93	+8.14			
Difference	1.60	9.40	8.58			
Gastrointestinal mass						
Light	−16.37	−4.16	−6.62	−15.23	−9.29	−4.17
Dark	−17.96	−9.40	−15.22	−19.84	−4.79	−21.01
Difference	1.59	5.24	8.60	4.61	4.50	16.84
Heart						
Light	+10.32	+7.66	+16.86	+4.69	−5.94	+11.91
Dark	+3.88	+1.62	+24.20	+2.08	+7.58	+15.56
Difference	6.44	6.04	7.34	2.61	13.52	3.65
Lungs						
Light	−9.81	−12.61	+13.91	−14.48	−28.51	+7.69
Dark	−10.21	−28.03	+12.51	−3.39	−24.43	+11.09
Difference	.40	15.42	1.40	11.09	4.08	3.40
Liver						
Light	−18.35	+3.61	+6.76	−18.10	−1.78	+9.62
Dark	−36.52	−20.41	−8.38	−39.63	−14.17	−12.98
Difference	18.17	24.02	15.14	21.53	12.39	22.60
Kidneys						
Light	−3.99	+8.50	+ .09	+1.27	+ .72	+3.48
Dark	−5.94	−5.87	−5.90	+2.74	+ .60	+7.74
Difference	1.95	14.37	5.81	1.47	.12	4.26
Brain						
Light	+1.27	−9.90	+ .72	−15.81	+3.48	−7.31
Dark	+2.74	− .24	+ .60	+5.46	+7.74	−2.56
Difference	1.47	9.66	.12	21.27	4.26	4.75

TABLE II—*Continued.*

Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Testicles						
Light	+6.48	+5.50	−2.98	−1.44	−8.63	−11.56
Dark	+4.78	−2.17	−2.83	+3.58	+3.71	−12.01
Difference	1.70	7.67	.15	5.02	12.34	.45
Spleen						
Light	−36.62	−32.48	−2.46	−38.72	−36.46	− .79
Dark	−28.38	−5.65	−10.89	−43.67	+2.38	−18.63
Difference	8.24	26.83	8.43	4.95	38.84	17.84
Thymus						
Light	+10.90	+25.13	−4.99	+15.76	+22.60	−7.90
Dark	+1.39	−13.64	−8.12	+ .52	−8.81	−24.66
Difference	9.51	38.77	3.13	15.24	31.41	16.76
Thyroid						
Light	+11.57	+89.21	−66.77	+32.17	+101.65	−22.54
Dark	+2.32	−37.52	−57.86	+27.15	+3.82	−18.00
Difference	9.25	126.73	8.91	5.02	97.83	4.54
Parathyroids						
Light	−13.81	−31.92	−24.35	−22.77	−44.38	−31.16
Dark	−15.19	−16.08	+30.91	−16.93	−9.34	+20.00
Difference	1.38	15.84	55.26	5.84	35.04	51.16
Hypophysis						
Light	−2.08	+2.69	+5.70	−3.62	−6.31	+ .78
Dark	+1.94	−7.59	−5.14	+2.31	−16.12	−13.21
Difference	4.02	10.28	10.84	5.93	9.81	13.99
Suprarenals						
Light	− .31	−9.00	−31.37	−9.62	−25.20	−38.12
Dark	+30.41	+5.11	+6.79	+29.43	+11.91	−5.28
Difference	30.72	14.11	38.16	39.05	37.11	32.84
Pineal						
Light	−6.44	−14.87	−26.55	+5.07	−21.98	−27.28
Dark	−1.27	−19.45	+14.59	+22.22	−15.36	+1.21
Difference	5.17	4.58	41.14	17.15	6.62	28.49

TABLE II—*Concluded.*

Organ	Actual weight			Relative weight		
	Group I	Group II	Group III	Group I	Group II	Group III
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Axillary lymph nodes						
Light	+37.04	+42.40	+24.00	+40.70	+35.47	+23.12
Dark	+11.75	+14.62	−6.51	+8.81	+18.80	−14.51
Difference	25.29	27.78	30.51	31.89	16.67	37.63
Popliteal lymph nodes						
Light	+7.88	+17.53	+23.80	+9.48	+12.76	+26.54
Dark	−7.97	+ .64	−8.29	−7.50	+6.25	−15.45
Difference	15.85	16.89	32.09	16.98	6.51	41.99
Mesenteric lymph nodes						
Light	−18.24	+ .84	−1.23	−21.01	−5.41	−4.73
Dark	−17.93	+5.74	+3.76	−24.35	+12.41	−6.76
Difference	.31	4.90	4.99	3.34	17.82	2.03
Deep' cervical lymph nodes						
Light	−1.38	+10.50	−34.40	−1.85	+7.08	−36.56
Dark	+4.24	−65.25	+2.90	−1.80	−64 60	−15.02
Difference	5.62	75.75	37.30	.05	71.68	21.54
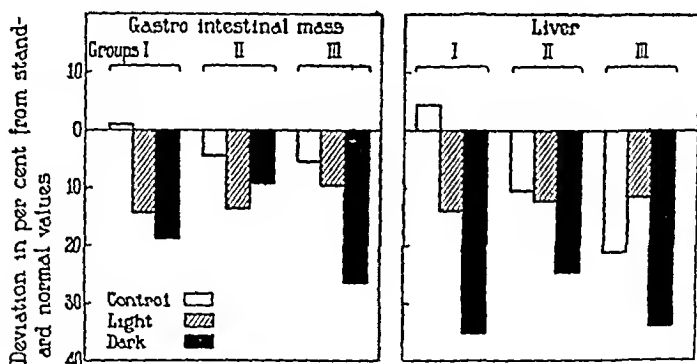
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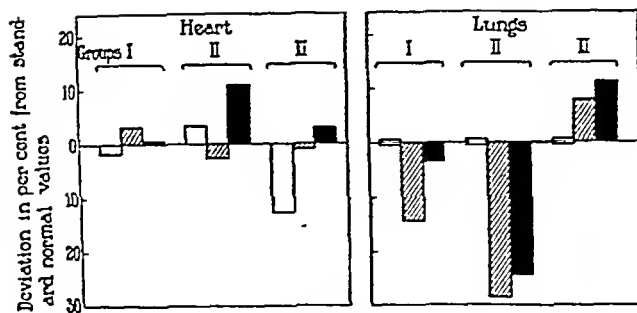
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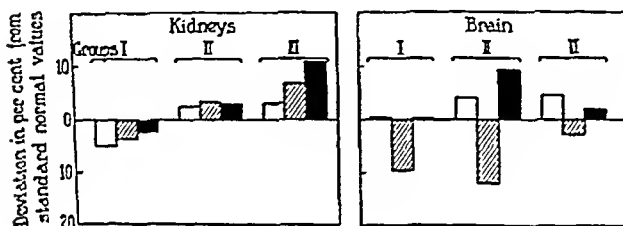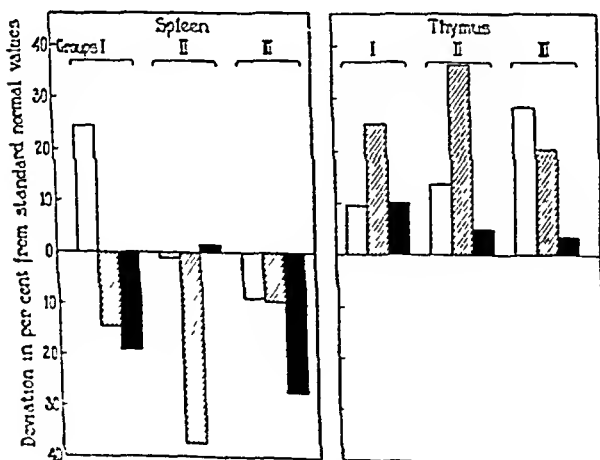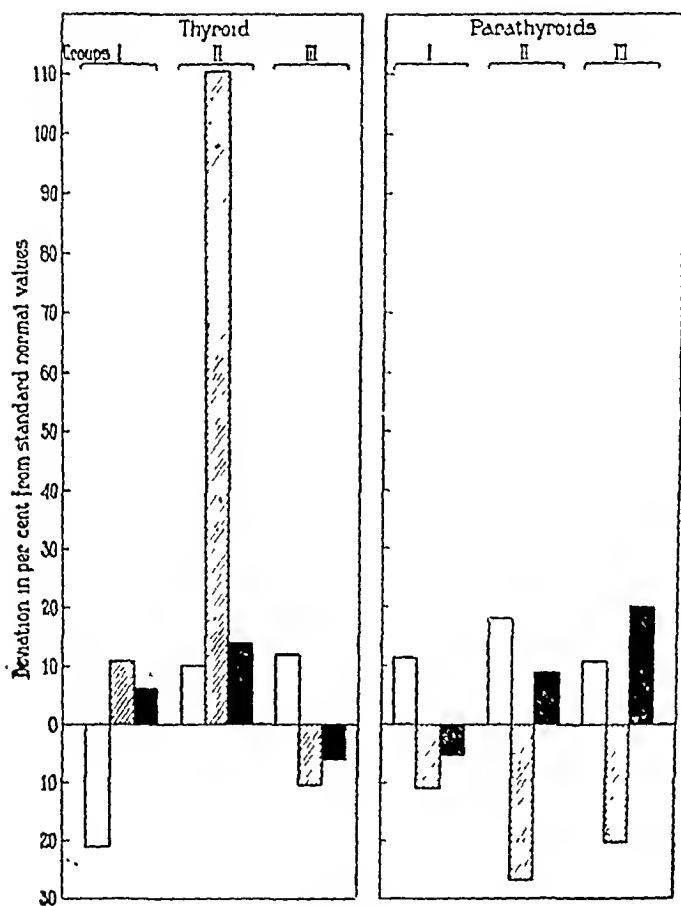
TEXT-FIG. 3.
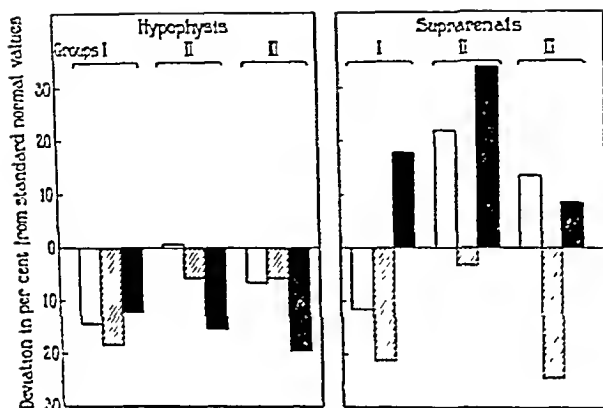
TEXT-FIG. 4.
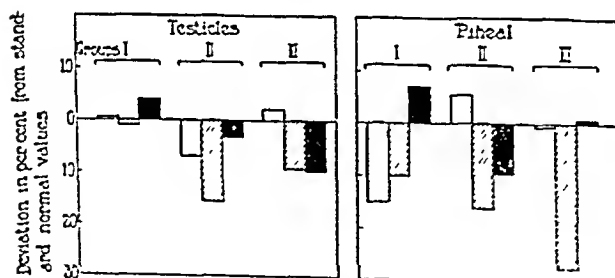
TEXT-FIG. 5.

TEXT-FIG. 6.

TEXT-FIG. 7.
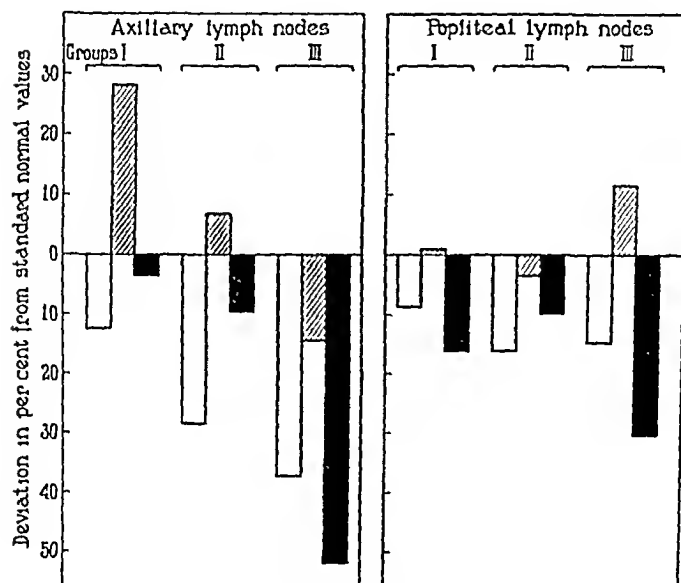
TEXT-FIG. 8.
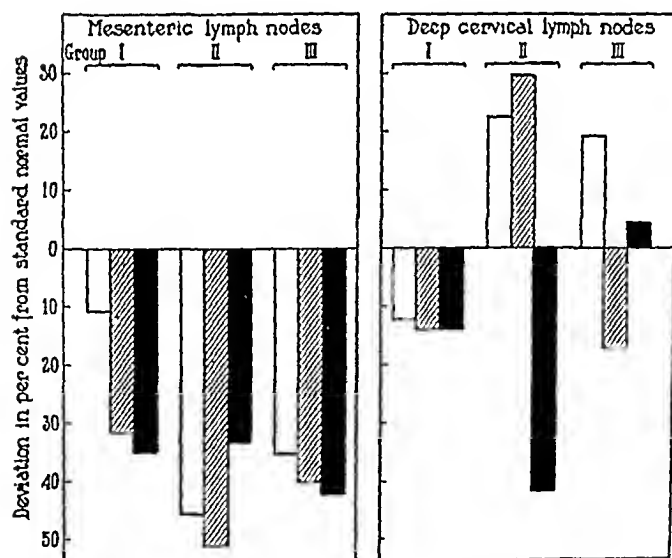
TEXT-FIG. 9.

DISCUSSION AND CONCLUSIONS.

In comparing the experimental results with standard values, some allowance must be made for inherent differences in material. The standard values represent a cross-section of the material used for ordinary experimental purposes in this laboratory over a period of $3\frac{1}{2}$ years, and this material included animals of various breeds, ages, and weights, but was composed mainly of gray and brown rabbits, 8 to 12 months old, which had been caged in the laboratory for 2 to 8 weeks. The animals of the present series were of a comparable age at the beginning of the experiments, but they had been caged in the laboratory approximately 8 months. Group I was composed entirely of white rabbits, Group II of black, while Group III contained animals of a dark color. The age difference is partially, if not entirely, compensated by the correction made for weight with the exception of the dark animals of Groups I and II which were undoubtedly older than the average animal of corresponding weight in the standard series.

A further distinction is to be made on the basis of the occurrence of periodic or cyclic variations in the weights of organs (6). The standard material was collected over a period of $3\frac{1}{2}$ years and included a wide range of spontaneous variations, while the experimental results represent values obtained at a particular time. In this connection, it should also be pointed out that some difference may be expected between the results for Groups I and II on the one hand and Group III on the other, due to the fact that the animals were killed at a time when, as a rule, the weights of organs are changing, and that there was an interval of 3 weeks between the killing of the first and last groups.

Considering the results recorded above, the points to be determined are, first, whether the results obtained show any material difference in the physical constitution of animals living under different environmental conditions, and, second, whether there is any evidence that the effects produced were influenced by the color or breed of the animals. In analyzing the results, Groups I and II are to be regarded as representing comparable environmental conditions; the status of Group III is problematic as the light and dark divisions of this group were interchanged twice during the experiment, and these animals were

returned to their original environmental conditions only 5 weeks before the experiment was concluded. The question is, therefore, whether the results obtained suggest a conformity with animals of the same class in Groups I and II or a reverse relation.

By reference to Tables I and II and Text-figs. 1 to 9, it will be seen that in most instances, the values obtained for light, dark, and control animals tend to preserve a common relation to the standard values, irrespective of differences in environmental conditions. It is evident, therefore, that the results are affected by the action of some factor which influences the weights of organs of all animals in a given manner, but not necessarily to the same degree. For example, the actual and relative weights of the gastrointestinal tract were subnormal for all except one lot of control animals (Tables I and II and Text-fig. 1). This is an almost constant finding for rabbits that have been caged in the laboratory for considerable periods of time. From a further analysis of the results, it will be seen, however, that in all cases the weights for light and dark animals were smaller than those for the corresponding controls (Text-fig. 1). The control animals of Groups I, II, and III showed a progressive decrease in the relative weight of the gastrointestinal mass, and the light groups a progressive increase, while the results for the 3 groups of animals in the dark were irregular.

A similar result was obtained for the liver (Text-fig. 1) as might be expected on account of the functional relation between the two organs. The progressive reduction in the weight of the liver of control animals was greater than that of the gastrointestinal mass, while the values for animals in the light remained virtually constant. Otherwise the results show an absolute agreement which is made particularly striking by the fact that the percentage values for light animals are practically the same for gastrointestinal mass and liver.

The results for the heart are irregular and show no clearly defined difference. There is merely a suggestion that the weight of the heart per kilo of body weight may have been greater for animals in the dark than for control and light groups (Text-fig. 2).

The significance of the results for the lungs is also uncertain. The weights for light and dark animals of Groups I and II were less than the controls but were greater for Group III. There is, however, a

consistent relation between the values for light and dark animals in that the value for animals in the dark is larger in all cases than that for animals in the light (Text-fig. 2).

The relative weights of the kidneys (Text-fig. 3) furnish an interesting example of an instance in which there is a remarkably close agreement between all values for a given group and only a slight difference between experimental and standard values with an extremely small but consistent difference between light and dark animals and the corresponding controls. The difference in weight is not sufficient in itself to be regarded as of any significance but the fact that the values for light and dark animals are larger than those for the controls in all cases and that those for animals in the dark tend to be larger than those for the light leads one to suspect that the results may·be significant. Attention may also be called to the fact that while all values for white animals are below normal, those for dark colored animals are above normal.

The results for the brain are of a similar order but with differences that are more clearly defined (Table I and Text-fig. 3). In the case of actual weights, the relative order of magnitude is control, light, and dark, but when the results are reduced to weights per kilo of body weight, the values for both control and dark animals are at or above normal, while those for light animals are below normal with a clear line of separation between. In this case, there is a further suggestion of a difference between white and black animals on the one hand, and Groups II and III on the other. This is seen chiefly in the results for the control and dark divisions of Groups I, II, and III and in the reduction of the deviation from normal of both the light and dark divisions of Group III as compared with Group II, while the controls remain constant (Text-fig. 3).

The values obtained for the actual weight of the spleen (Table I) show that this organ was distinctly larger in control than in light and dark animals. There is also evidence that it was larger in white than in animals of dark color. The smallest values for Groups I and II were given by animals living in the light. In Group III, the smallest value was given by animals of the dark division. But if we consider the order of magnitude of the values for light and dark divisions of the 3 groups, there is a suggestion that the figures for Group III show a

reversed relation due to the persistence of an effect which was not abolished during the last 5 weeks of the experiment.   The results for relative weight (Text-fig. 4) show essentially the same relations, but they are not brought out so clearly as they are by a comparison of actual weights.

The conditions shown by the thymus are just the reverse of those shown by the spleen in so far as the relative magnitudes of the values for different classes of animals are concerned.   This is, of course, to be expected on account of the relation that obtains between these two organs.

The results for the thyroid are very striking (Table I and Text-fig. 5).   In the first place, the results for control animals show that there is unquestionably a difference in the weight of the thyroid of white and of black or dark colored animals.   This difference holds also for the light and dark divisions of Groups I and II, but not for Group III. In the second place, the animals of the light divisions of Groups I and II gave the highest values and the controls the lowest, while in Group III this relation was reversed.   The very high value obtained for the light animals of Group II represents a condition shown by 4 of the 5 rabbits comprising this division and is, therefore, a valid result.   The reduction in the weight of the thyroid shown by the light and dark divisions of Group III is difficult to account for, but may be a result of recent changes in environmental conditions.

The parathyroids show a clear separation according to environmental conditions.   Actual and relative weights for animals in the light are all subnormal and smaller than those for the corresponding dark divisions, while the values for the controls are above normal and in 2 of the 3 groups are larger than those for dark animals (Tables I and II and Text-fig. 5).   There is also a suggestion that the effect produced on white rabbits (Group I) by living in an environment of neon light or in the dark is less than that shown by dark colored animals (Groups II and III).

The most interesting feature of the results obtained for the hypophysis is the difference between Group I on the one hand, and Groups II and III on the other (Table I and Text-fig. 6).   The light and dark divisions of Groups II and III show the same relation to each other, while in Group I this relation is reversed.   Group I also shows the

smallest values for control and light animals, while in Groups II and III animals of these classes give the largest values.

The suprarenals show a similar relation, so far as control animals are concerned, with a distinction between light and dark divisions that is clearly defined in all cases (Table I and Text-fig. 6). There is the suggestion here also that the smaller values for Group III as compared with Group II may have been due to some condition affecting control as well as experimental animals and, in the case of the dark division, to the persistence of an effect from the previous exposure to neon light.

The results for the testicles and pineal gland are irregular and of uncertain significance (Table I, Text-fig. 7). On the whole, it seems, however, that there is evidence of a tendency toward smaller values for animals in the light as compared with either the controls or the dark divisions of the same group and for the dark to be larger than the controls. It is the variation of results for control animals that is confusing.

The axillary and popliteal lymph nodes may be considered together as they are parts of the same system of organs (Table I, Text-fig. 8). The results obtained for the two groups of nodes agree in most respects. The animals in the light gave the largest values in all cases; the weights were either close to normal or above normal, while those of control and dark animals were subnormal with a relation between the two that was inconstant.

The deep lymph nodes, including the central mesenteric and deep cervical groups, show an entirely different situation (Table I and Text-fig. 9). The mesenteric nodes were extremely small in all cases, and in 8 of the 9 divisions the relations found agreed closely with those shown by the gastrointestinal mass. This agreement is to be expected as the weights of the two organs usually vary in the same direction.

The values for the deep cervical lymph nodes are of doubtful significance as several animals had infections of the nasal sinuses. The results are of interest, however, as an illustration of the disturbing influence of an extraneous factor.

The results have not been subjected to a detailed statistical analysis as the number of animals in each division (5) is too small to warrant such treatment. Reference to Table II will show, however, that in

many cases the differences found are sufficiently large to be regarded as significant when taken in conjunction with the trend of the apparent effect produced by a given type of environment.

Thus far, we have considered the results obtained for the weight of a given organ independent of the weight of any other organ, but it is evident that even large variations in the weights of organs may occur without materially changing the relations between organs, or that the relation of the weight of one organ to that of another may be greatly altered by slight changes in the weights of the organs concerned. This aspect of the problem of organic constitution is of even greater importance than the actual weights of organs or the weight of organs per unit of body weight, but we cannot undertake a detailed discussion of the results from this point of view as there are so many comparisons that might be made. A few examples will serve to show that the relations between organs were affected.

From the point of view of nutrition and growth the relation between the liver and the gastrointestinal mass is of interest. Normally, the weight of the liver per kilo of body weight is 1/5 or 20.00 per cent of that of the gastrointestinal mass (mean standard value, 20.41 per cent) and this relation is affected very little by differences in body weight *per se*. In these experiments, the ratio between the weight of the liver per kilo of body weight and that of the gastrointestinal mass, expressed in per cent, was as follows:

	Group I	Group II	Group III	Mean
Control	20.70	18.81	16.72	18.74
Light	18.06	18.30	17.60	17.98
Dark	16.27	16.98	18.30	17.18

The important points to be noted are the magnitude and the constancy of the values obtained. Thus the results for control animals were somewhat irregular, while the values for the light animals of Groups I and II were more constant and slightly smaller and those for the dark animals were still smaller. The values for Group III were apparently affected by the final change in environment as the figure for the dark division of this group is in absolute agreement with the

light division of Group II, while the figure for the light division is nearer that obtained for animals in the dark.

From the standpoint of metabolism, the relation between the thyroid and suprarenals is also of interest, as has been shown by Marine and his associates (7). Unlike the liver and gastrointestinal mass, the ratio between thyroid and suprarenal weights in normal rabbits is inconstant. The mean standard value (relative weights) is 60.76 per cent, but this value is subject to wide variation, as are the weights of the thyroid and suprarenals. The range of variation found on the basis of a body weight grouping of the normal rabbits studied by us was 47.94 to 77.35 per cent or a deviation of approximately 25.00 per cent in either direction from the mean value. The values obtained in these experiments were as follows:

	Group I	Group II	Group III	Mean
Control	63.49	63.79	69.54	65.61
Light	87.93	136.25	73.94	99.37
Dark	64.41	60.83	61.92	62.39

It is evident that these figures indicate a distinct difference in the thyroid-suprarenal equilibrium of the 3 classes of animals. The figure for the light division of the third group is again suggestive of the persistence of an effect following the change of environmental conditions, and there is also a suggestion of a difference between the conditions presented by white and by black animals of the light and dark divisions.

The values for the ratio of the hypophysis to the thyroid and for the thyroid to the thymus, and for the parathyroids to the thyroid are tabulated below as further examples of the effect of environmental conditions on organic equilibria.

Ratio of the Hypophysis to the Thyroid in Per Cent.

	Group I	Group II	Group III	Mean
Control	8.57	7.23	6.61	7.47
Light	6.48	3.92	9.27	6.56
Dark	8.34	8 71	8.55	8.53

Ratio of the Thyroid to the Thymus in Per Cent.

	Group I	Group II	Group III	Mean
Control	11.33	15.20	13.69	13.41
Light	15.64	27.35	13.07	18.70
Dark	11.56	13.00	10.85	11.80

Ratio of the Parathyroids to the Thyroid in Per Cent.

	Group I	Group II	Group III	Mean
Control	4.39	3.38	3.12	3.63
Light	3.80	1.65	4.25	3.23
Dark	4.13	4.41	5.91	4.82

Similar differences are shown by other organs. It is thus evident that the conditions under which the animals lived affected not only the weights of organs but the organic equilibrium in the broadest sense. Moreover, while the number of animals is too small to warrant a definite conclusion, it appears that there are some constitutional differences between black and white rabbits and that the two classes of animals do not show the same response to changes in environmental conditions. The results for the light and dark divisions of Group III do not agree in all respects with those for the corresponding divisions of Groups I and II; in some instances the results are reversed which suggests that the condition found at autopsy represents a transitional state referable to a change in environmental conditions.

In the present state of knowledge, it is difficult to correlate the size or weight of a given organ with a particular state of functional activity. This difficulty is increased by the fact that under certain circumstances, the relation may be direct, while under others it is inverse. Still, with due allowance for any uncertainty that may exist, there is sufficient evidence to warrant the conclusion that in these experiments there is a relation between the results obtained for organ weights on the one hand, and for increase in body weight and the growth of hair on the other. This is best shown by the results for the liver and for the ratio of the thyroid to the suprarenals. The results obtained for the thyroid-suprarenal relation agree with theoretical

expectations based on the work of Marine and his associates (7) and the results for the liver follow as a logical consequence of the activity of these two organs.

SUMMARY.

The influence of light environment on the organic constitution of normal rabbits was studied by comparing the weights of organs of animals that had been living under cerrtain conditions for long periods of time.

It was found that the light environment produced an effect on the physical constitution of the rabbits which was comparable to the effects produced on the functional activity of the same animals.

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CHEMICAL FINDINGS IN THE BLOOD OF THE DOG AFTER TEMPORARY OBSTRUCTION OF THE PYLORUS.

By RUSSELL L. HADEN, M.D., AND THOMAS G. ORR, M.D.

(*From the University of Kansas, School of Medicine, Kansas City, Kansas.*)

MacCallum and his coworkers (1), and Hastings, Murray, and Murray (2) in studying pyloric obstruction in the dog noted a marked fall in chlorides and a rise in the CO_2 combining power of the blood. Similar observations in clinical cases were made by Grant (3). The close similarity of such changes with those found in high intestinal obstruction (4) led us to study the non-protein nitrogen of the blood in dogs with the pylorus obstructed (5). The non-protein nitrogen in such animals was found to reach the high levels characteristic of high intestinal obstruction. These observations have since been verified by others in both clinical (6) and experimental work (7, 8).

Our special interest in the subject was the relation of the chloride metabolism to the toxemia as reflected by the non-protein nitrogen level. The observation was made that the toxemia and rise in non-protein nitrogen in high intestinal obstruction do not usually occur until the chloride store of the blood is reduced below a certain level . (4). It was also shown that the administration of sodium chloride had a remarkably beneficial effect on the toxemia of intestinal and pyloric obstruction and likewise in preventing the onset of a toxemia if given early (9). No beneficial effects were observed from water alone, other inorganic salts, or glucose. The therapeutic value of NaCl is now well accepted in both pyloric and intestinal obstruction. None of the explanations given for its favorable action is entirely satisfactory.

With the vomiting incident to pyloric obstruction much water and chloride are lost. This dehydration causes a diminution in kidney function with consequent retention of the end-products of protein metabolism. In the toxemias due to upper gastrointestinal tract

obstruction, there is a markedly accelerated nitrogen metabolism with increased tissue breakdown (10, 11). The rise in the non-protein nitrogen in the blood is often too rapid to be accounted for by simple retention. The high level of non-protein nitrogen seems due to both retention and increased tissue destruction.

The exact relation of the chlorides to the toxemia and tissue destruction is as yet undetermined. We suggested originally the possibility that sodium chloride may directly neutralize the bodies responsible for the toxemia. This hypothesis was based on the seeming greater loss of chloride from the blood than could be accounted for in the vomitus and urine and on the striking effect of sodium chloride in relieving and preventing the toxemia. Further studies by Gamble and McIver (8), White and Bridge (12), and Atchley and Benedict (13) indicate that all the chloride lost from the blood and tissues is found in the urine and gastric and intestinal secretions. There is also a fall in the chloride of the tissues (12, 14), and during the toxemia very little chloride is excreted in the urine. These facts render our original theory unlikely.

This still however does not throw any light on the problem of the relation of the chloride level to the tissue metabolism. The proper amount of chloride is necessary to maintain water balance and kidney function. Autolysis may conceivably be more rapid in tissue with a low chloride content. It is also possible that the tissues may be more easily injured by circulating toxins, if such be present, when the salt content is low. These experiments have been made in an attempt to throw some light on the exact relation of the chloride level to the toxemia of upper gastrointestinal tract obstruction.

In studying the relation of the sodium chloride to the blood chemical changes and toxemia, it is most desirable to be able to produce experimentally the characteristic blood changes and toxemia without having to consider mechanical factors, the possibility of infection, and operative risk. We have found that such a condition can be produced by ligating the pylorus and releasing the obstruction in 24 to 72 hours. The thick wall of the pylorus precludes the possibility of rupture of the viscus as frequently happens after ligation of the intestine. The obstruction is easily eliminated by removing the ligature under local anesthesia with little operative risk. In some of the surviving animals

there is produced a characteristic toxemia in which mechanical factors are eliminated making an ideal condition for the study of the blood chemical changes.

Method.

All experiments have been made on dogs. All primary operations were done under ether anesthesia with aseptic technique. Obstruction of the pylorus was

TABLE I.

Typical Changes in Blood of Dog after Simple Obstruction of Pylorus.

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO₂ combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. %	
1	0	35.7	11.2	460	24.0	Pylorus obstructed
	1	46.0	14.5	320	52.0	
	2	111.0	49.0	230	37.7	Died
2	0	37.5	7.9	500	35.4	Pylorus obstructed
	1	38.9	15.4	470	30.5	
	2	57.3	30.8	360	50.2	
	3	129.0	70.0	310	56.0	Died
3	0	28.9	12.6	490	27.5	Pylorus obstructed
	1	27.0	11.2	450	44.7	
	2	61.0	25.2	380	42.8	
	3	76.5	28.7	340	53.9	
	4	181.0	128.2	260	45.3	Died
4	0	33.7	12.6	500	30.5	Pylorus obstructed
	1	46.0	21.0	480	36.2	
	2	155.0	47.6	330	29.4	Died

effected by ligating with a piece of narrow tape. The tape was fixed to the anterior abdominal wall so that it could be easily located at the second operation. The obstruction was released by making a short incision under local anesthesia, cutting and removing the tape. At autopsy after such a procedure no permanent anatomic changes in the pylorus could be observed. The most desirable time for the second operation is usually 48 hours after the obstruction is made. The dogs were kept in metabolism cages. No food was given during the experiment and usually none for 48 hours before operation. Water was allowed *ad libitum*.

TABLE II.

Course of Toxemia in Dogs Recovering after Temporary Obstruction of Pylorus without Treatment.

Dog No.		Blood				Remarks
		Amount per 100 cc.			CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. %	
5	0	37.5	10.3	480	30.5	Pylorus obstructed
	1	34.5	8.4	440	40.9	
	2	61.0	26.6	350		Obstruction released
	3	79.0	30.1	310	54.9	
	4	50.0	9.8	330	36.2	
	5	27.3	18.9,	330	40.0	
	6	26.3	15.4	370	56.0	
	7	30.0	9.1	360	40.9	
	8	31.2	10.7	380	49.4	
	9	30.3	6.5	430	42.8	
	10	27.3	7.5	460	48.5	Well
6	0	27.7	13.1	460	41.9	Pylorus obstructed
	1	37.5	14.7	370	50.4	
	2	65.5	30.8	330	58.7	
	3	42.8	14.0	330	56.8	
	4	40.0	16.8	340	59.6	
	5	39.5	16.8	340	56.5	
	6	40.0	18.2	340	47.5	
	7	37.5	17.5	350	55.8	
	9	38.0	9.1	370	57.7	
	10	30.9	8.4	400	52.2	
	11	33.0	10.7	420	48.5	
	12	57.3	7.5	450	51.3	Well
7	0	28.5	13.1	500	38.1	Pylorus obstructed
	1	25.9	9.8	440		
	2	30.0	9.1	440	41.9	
	3	33.0	16.1	380	40.0	Obstruction released
	4	50.8	22.9	330	53.2	
	5	34.5	20.7	370	61.7	
	6	42.8	23.7	370	57.9	
	7	125.0	73.6	330	57.7	
	8	32.3	9.1	380	53.9	
	9	31.6	16.1	410	50.2	
	10	42.2	14.0	390	67.1	
	11	32.3	11.7	420	57.7	
	12	41.0	15.4	400	46.6	
	13	50.0	13.1	420	58.7	Well

TABLE II—*Concluded.*

Dog No.	Blood					Remarks
	Amount per 100 cc.				CO_2 combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	mg.	mg.	vol. %	
8	0	24.6	7.7	450	37.2	Pylorus obstructed
	1	28.0	16.8	400	40.0	
	2	89.0	52.5	330	41.0	Obstruction released
	3	72.3	40.6	320	38.1	
	4	34.5	17.5	380	36.2	
	6	40.0	18.2	420	35.3	
	7	38.0	16.1	400	31.5	
	8	34.5	14.7	420	43.8	
	9	30.0	9.1	440	39.0	
	10	29.4	17.5	440	41.9	Well

Blood was obtained for analysis from the jugular vein just before operation and at 24-hour intervals thereafter.

The non-protein nitrogen of the blood was determined by the method of Folin and Wu (15), urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (16), CO_2 combining power by the method of Van Slyke (17), and the chlorides in the tungstic acid filtrate after the technique suggested by Gettler (18). For the non-protein nitrogen in the urine Folin's micro method (19) was used, and for the chlorides a modified Volhard-Arnold method.

EXPERIMENTAL OBSERVATIONS.

The blood chemical changes in four dogs in which the obstruction was not released are shown in Table I. These all show the characteristic findings, namely, a fall in chloride and a rise in CO_2 combining power, and in non-protein and urea nitrogen. The animals died in 48 to 96 hours after operation.

In eight dogs the obstruction was released but no treatment was given. Four recovered, and four died. In the animals recovering (Table II) there was a gradual rise in the blood chloride until the normal level was reached. No food was given during the experiment so it is quite evident that there was a shift in the store of body chloride so that the diminished supply of blood chloride was restored. The four dogs which died showed a progressive fall in chloride and rise in

TABLE III.

Effect of Sodium Chloride Given by Mouth on Toxemia Induced by Temporary Obstruction of Pylorus.

Dog No.	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Remarks
		ms.	ms.	ms.	vol. %	
9	0	34.9	15.4	430	32.4	Pylorus obstructed
	2	31.6	14.0	330	46.6	
	3	30.0	16.1	300	60.0	Obstruction released
	4	33.3	17.5	310	50.2	
	5	37.5	20.3	290	55.0	
	6	42.2	12.6	320	53.6	
	7	60.0	18.2	360	59.6	
	9	79.0	35.0	360	40.0	
	10	67.5	37.8	320	52.0	10 gm. NaCl by mouth
	11	38.9	21.0	350	45.7	10 gm. NaCl by mouth
	12	23.3	10.5	470	41.9	10 gm. NaCl by mouth
	13	25.7	15.4	500	41.9	Well
10	0	30.9	14.0	470	28.7	Pylorus obstructed
	1	31.6	13.8	450	38.1	
	2	39.5	14.0	360	39.0	
	3	111.0	52.5	300	53.9	Obstruction released
	4	67.0	39.3	350	45.7	
	5	60.0	36.4	310	46.6	
	6	55.0	30.8	340	47.5	
	7	51.8	31.5	350	53.0	
	8	70.0	38.5	340	52.2	
	9	72.8	48.3	340	52.0	
	10	79.0	49.0	370	50.2	10 gm. NaCl by mouth
	11	34.1	21.7	390	57.7	10 gm. NaCl by mouth
	12	34.9	20.3	470	39.0	
	13	34.1	19.6	470	52.0	
	14	33.0	19.6	500	48.5	
	15	39.5	23.8	470	50.2	
	16	28.5	16.1	520	46.6	
	17	27.0	17.5	600	40.0	Well
11	0	61.0	26.6	510	30.5	Plyorus obstructed
	1	68.8	35.0	500	38.1	
	2	117.0	51.1	400	59.6	Obstruction released
	3	137.0	75.7	370	55.8	
	4	123.0	56.7	350	49.4	
	5	120.0	60.2	380	45.7	
	6	120.0	64.4	400	51.3	
	7	100.0	55.3	400	53.9	

TABLE III—*Concluded.*

Dog No.		Blood				Remarks
		Amount per 100 cc.			CO₂ combining power	
	Day after operation	Total non-protein nitrogen	Urea nitrogen	Chlorides		
		mg.	*mg.*	*mg.*	*vol. %*	
12	8	103.0	59.5	380	51.3	
	9	115.0	57.4	390	47.4	
	10	98.8	45.5	400	55.8	
	11	143.0	82.9	380	28.7	
	12	100.0	50.4	420	45.7	
	13	126.0	60.2	400	36.2	10 gm. NaCl by mouth
	14	88.4	44.1	480	4.0	10 gm. NaCl by mouth
	15	60.0	23.1	440	30.5	
	16	71.3	43.9	530	32.4	
	17	57.3	35.0	570	31.5	
	18	42.2	15.4	580	24.0	
	19	35.7	14.0	520	28.7	
	20	38.9	19.6	480	30.5	Well
13	0	51.8	26.2	430	42.1	Pylorus obstructed
	1	30.3	12.6	340	67.1	
	2	68.2	43.3	210	72.7	Obstruction released
	3	132.2	72.9	260	74.5	
	4	83.3	57.1	230	80.1	
	5	77.3	38.2	270	74.5	
	6	107.0	63.9	260	74.5	
	7	158.0	98.8	350	56.9	
	8	208.0	123.3	290	58.8	
	9	173.0	100.8	250	67.5	
	10	230.0	141.5	270	65.3 ·	
	11	250.0	159.7	270	71.8	10 gm. NaCl by mouth
	12	136.0	78.5	410	61.6	Died immediately after intravenous injection of 25 per cent NaCl

non-protein nitrogen after the obstruction was released. The course of the toxemia was not influenced by the release of the obstruction.

Five animals were treated with sodium chloride by mouth after a toxemia had been established for a sufficiently long period to indicate that it would not adjust itself spontaneously (Tables III and IV). The sodium chloride was given by mouth in 1 gm. tablets with a small

TABLE IV.

Blood and Urine Findings after Temporary Obstruction of the Pylorus.

Dog No.	Day after operation	Blood					Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Hematocrit (% plasma)	Amt.	Chlorides		Nitrogen		
		mg.	mg.	ms.	vol. %	%	cc.	%	gm.	%	gm.	
14	0	44.0	10.3	470	38.1	51						Operation
	1	28.0	9.3	430	45.7	41						
	2	125.0	36.0	470	49.4	42	250	0.50	1.50	0.96	2.40	Obstruction re-
	3	175.0	92.2	450	50.2	43	900	0.01	0.09	0.33	2.97	leased
	4	276.0	126.6	380	40.0		320	0.01	0.032	1.38	4.4	
	5	220.0	132.6	370	40.9	53	680	0.01	0.068	0.44	2.99	
	6 a.m.	236.0	154.1	370	41.9	58	560	0.22	1.23	0.68	3.80	100 cc. 10 per cent
	6 p.m.	169.0	98.5	430	38.1	59						NaCl by stom-
	7	78.0	57.0	490	38.1	61	1330	0.63	8.38	0.68	9.0	ach tube
	8	75.0	47.2	450	32.4	67	270	0.11	0.30	2.00	5.4	
	9	27.7	17.3	490	38.1	65	240	0.06	0.14	2.50	6.0	
	10	24.4	14.9	480	38.1	72	140	0.07	0.09	3.30	4.6	
	11						190	0.15	0.29	2.85	5.4	
	12	28.1	8.9	580	34.4		150	0.35	0.53	2.00	3.0	Recovered
	13											
15	0	33.0	8.4	470	34.3	55						Operation
	2	68.3	33.6	420	48.3	42						
	3	132.0	98.1	430	50.2	52	730	0.35	2.56	0.64	4.67	Obstruction re-
	4	173.0	112.8	320	47.5	48	340	0.07	0.24	1.77	6.02	leased
	5	159.0	91.1	440	41.6		275	0.05	0.14	3.00	8.25	
	6						355			3.00	10.65	
	7		44.8	410	45.7		195	0.03	0.06	3.00	5.05	
	8	37.5	23.8	410	52.0		220	0.03	0.06	1.02	4.22	
	9	40.6	16.8	410	55.8		220					
	10	29.1	14.0	450	59.6		210	0.05	0.11	1.00	2.1	Recovered
16	0	30.0	13.3	490	38.1							Operation
	1	30.3	12.4	420	41.9							
	2	59.5	38.5	330	57.7		1000	0.05	0.50	0.92	9.2	Obstruction re-
	3	41.6	15.4	390	52.0		625	0.05	0.31	1.23	7.7	leased
	4	41.0	22.4	380	48.8		610	0.05	0.30	1.04	6.3	
	5	32.6	16.1	380	52.0		500	0.02	0.10	1.92	9.6	
	6	38.9	14.0	390	50.2		235	0.03	0.70	3.00	7.05	
	7	31.9	15.4	400	55.8		365	0.06	0.22	1.44	5.3	Recovered
	8	27.0	16.8	400	54.6		265	0.03	0.08	1.10	2.9	

amount of water in four animals and in 10 per cent solution in another. A very large amount of salt was required for each animal. The four recovering showed a rapid return of blood findings to normal, and a disappearance of the signs of toxemia. The animal which did not recover showed a marked fall in non-protein nitrogen after the administration of 10 gm. of salt by mouth. The following morning an intravenous injection of 25 per cent sodium chloride was attempted with immediate death of the animal.

In three animals chemical studies were also made on the urine after the pyloric obstruction was released (Table IV). After the release there was no vomiting, so accurate urine collections could be made. Distilled water was allowed *ad libitum*. No food was given. In one animal (No. 13) the non-protein nitrogen continued to rise and the chlorides to fall after the removal of the obstruction. There was a marked diuresis, with a high nitrogen and low chloride excretion. Even with this marked diuresis and high nitrogen output the non-protein nitrogen in the blood continued to rise indicating the continuation of the increased protein destruction. The hematocrit reading before operation was 51, and 4 days after the release operation it was 58. When the non-protein nitrogen was 236 mg. per 100 cc. the animal was given 100 cc. 10 per cent sodium chloride by stomach tube in broken doses over several hours time. In the afternoon the non-protein nitrogen had fallen to 169 mg. with practically no change in the hematocrit reading. The following day there was a marked diuresis with a high nitrogen excretion. Almost all the chloride given was excreted within 48 hours. The non-protein nitrogen rapidly returned to normal and remained so. In two other animals there was also a marked diuresis after release of the obstruction. The nitrogen excretion was very high. The non-protein nitrogen returned to normal without treatment. The blood chloride returned to normal in one animal and almost to normal in the other.

DISCUSSION.

Temporary obstruction of the pylorus is a procedure by which the characteristic toxemia of upper gastrointestinal tract obstruction may be produced without having to consider mechanical factors, infection, or operative risk. It gives an opportunity for studying the toxemia factor alone.

The animals which recovered after the release of the obstruction showed the findings now known as characteristic of pyloric obstruction. The animals which recovered spontaneously all showed a quite low blood chloride at the height of the toxemia. The lowest level of chloride always occurred with the highest non-protein nitrogen. As the non-protein nitrogen returned to normal the chloride rose and gradually returned to normal with the animal receiving nothing by mouth except distilled water. These results can be explained only on the basis of a redistribution of the body chlorides. Some of the chloride lost from the blood must have been stored somewhere within the body only to return to the blood after the toxemia is relieved. The level of chloride in the blood is evidently not a true index of the chloride in the body.

The effect of sodium chloride given by mouth in the toxic animals was immediate and most striking. In no instance was the salt given until it was quite apparent that the toxemia would not be relieved by water alone. In every animal so treated there was a rapid return of the blood to normal except the one in which a fatal result followed a later intravenous injection.

The study of the urine after temporary obstruction of the pylorus emphasizes again the high nitrogen excretion which can be explained only on the basis of increased protein destruction. There is a marked diuresis after the obstruction is released probably due to the increased urea content of the blood. The administration of sodium chloride caused a transient increase in the diuresis and output of nitrogen and salt with a rapid return to normal. The assumption seems justified that the restoration of the chlorides has stopped the increased protein destruction.

CONCLUSIONS.

The toxemia characteristic of upper gastrointestinal tract obstruction may be produced by temporary obstruction of the pylorus. This procedure affords an opportunity for studying the toxemia in the absence of mechanical factors, operative risk, and infection.

Animals which spontaneously recover from the toxemia may show a return of the blood chloride to normal when only distilled water is given. In such instances there must be a redistribution of the body store of chlorides.

The administration of sodium chloride by mouth to animals which show a toxemia without evidence of spontaneous recovery causes a rapid return of the blood to normal.

There is a marked diuresis with high nitrogen excretion during the toxemia. This is evidently due to the increased protein destruction.

The return of the blood chlorides to normal causes a cessation of the increased protein destruction.

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VIRUS III ENCEPHALITIS.

By THOMAS M. RIVERS, M.D., AND FRED W. STEWART, M.D.

(From the Hospital and Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 12 TO 14.

In 1923 Rivers and Tillett (1) described reactions in rabbits which were considered to be induced by the virus of varicella. Subsequently, however, in Rivers' (2, 3) laboratory and also in Swift's (4, 5), it was found that the reactions were caused not by the virus of varicella but by an unknown virus indigenous to rabbits which had been accidently encountered in the work on chicken-pox and rheumatic fever. Inasmuch as the spontaneous disease caused by the virus has not been recognized, no name other than Virus III has as yet been given this active agent. The term is used merely for convenience and designates the third strain of the virus encountered with which most of the work happens to have been conducted.

The character of Virus III lesions and the presence of acidophilic nuclear inclusions (3) in the injured tissues led Rivers and Tillett to do cross-immunity experiments to determine what relationship, if any, Virus III bears to herpetic virus (2). None was found. Furthermore, in 1924, rabbits were inoculated intracerebrally with Virus III to determine if it was capable of producing an encephalitis (2). A temperature above 104°F., which persisted for a week and which was much more marked in the experimental than in the control animals, was the only abnormality noted. Because of the mild reaction no further study of Virus III encephalitis was made at that time.

From the time of discovery of Virus III, early in 1923, until September, 1926, when emulsions of tissues containing the virus were frozen, desiccated, sealed in tubes, and stored on ice, testicular passages (approximately 300) were made at intervals of 3 or 4 days. In January, 1928, the dried virus was removed from the ice box and testicular passages were resumed. In the course of some experiments,

intracerebral inoculations with the virus were made in rabbits and signs of encephalitis, which were followed by death in a number of instances, were observed. It is with this encephalitis caused by Virus III that the present paper deals.

Methods and Materials.—In sealed tubes on ice, frozen and desiccated Virus III (6) retains its activity indefinitely. Its activity is also maintained for at least 6 weeks if infected testicular emulsions are mixed with equal amounts of glycerol, sealed, and stored on ice. Experiments, however, were always conducted with fresh material. Either emulsions of infected testicles or brain emulsions containing the virus served this purpose. The emulsions were prepared by grinding the infected tissues with sand in a mortar and then adding enough Locke's solution to make a 20 per cent suspension. To free the material from sand, centrifugation at low speed for 1 minute was employed. 2,000 gm. rabbits were used. 0.2 cc. of an emulsion was the amount chosen for intracerebral or for intradermal inoculation, and 1.0 cc. for intratesticular inoculation. Even though it does not so appear in the text-figures, at least two rabbits were inoculated each time the virus was passed. In working with Virus III this is necessary because an immune animal is occasionally encountered which results in the loss of the virus. The sterility of all tissues was tested by means of aerobic and anaerobic cultures. All operations were performed under ether anesthesia. Tissues for histological studies were fixed in Zenker's fluid and stained either with eosin-methylene blue or by Giemsa's method.

In Text-figs. 1 to 3 are outlined the methods of procedure employed in the study of Virus III encephalitis. Although the majority of intracerebral inoculations was made with testicular virus, it will be seen in Text-fig. 1 that the virus propagated itself through three successive intracerebral passages, causing in each instance definite signs of encephalitis, *e.g.*, tremor, ataxia, irritability, circling, salivation, retention of urine, generalized tonic and clonic contractions of the skeletal muscles, or paralysis. From the text-figures it will also be observed that potent testicular virus did not produce signs of encephalitis in every rabbit of certain series and that the results of intracerebral inoculations varied considerably in the different series of animals, *i.e.*, at times all the rabbits died, while on other occasions none showed signs other than a pathological increase in temperature. There is no adequate explanation for this remarkable variation in activity of the virus when inoculated intracerebrally.
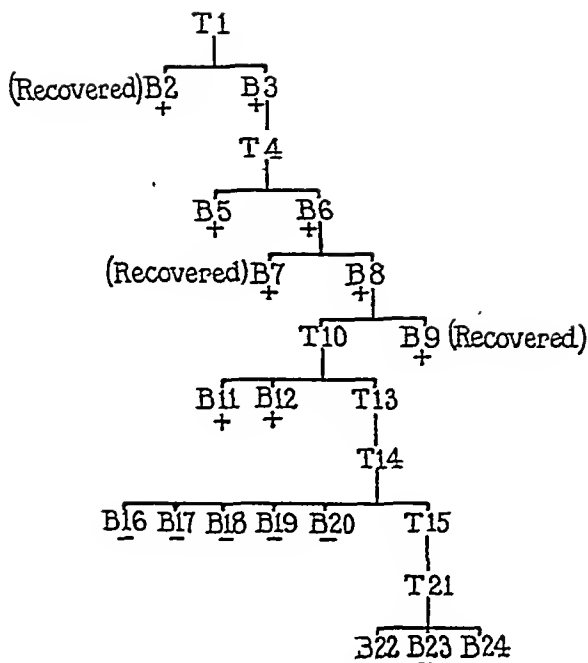
TEXT-FIG. 1. Outline of procedure employed in the study of Virus III encephalitis. T indicates site of inoculation (testicle) and also organ emulsion (testicle) used for next passage of virus. B indicates site of inoculation (brain) and also organ emulsion (brain) used for next passage of virus. $+$ indicates occurrence of definite clinical signs of encephalitis. $-$ indicates absence of clinical signs of encephalitis other than fever. Rabbit T 1 was inoculated intratesticularly with glycerolated Virus III 5 passages removed from an animal inoculated with the desiccated material that had been stored on ice more than a year.
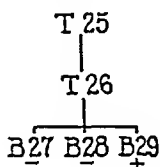
TEXT-FIG. 2.
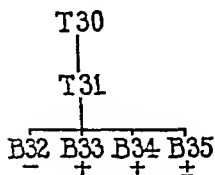
TEXT-FIG. 3.

TEXT-FIG. 2. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 25 was inoculated intratesticularly with glycerolated Virus III from Rabbit T 1.

TEXT-FIG. 3. Symbols employed in manner similar to that in Text-fig. 1. Rabbit T 30 was inoculated intratesticularly with desiccated Virus III that had been stored on ice more than a year.

Rabbit B 5.

March 6. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 4.

March 7. Temperature 104.2°; animal appears normal. March 8. Temperature 105.0°; animal appears normal. March 9. Temperature 103.4°; sick and irritable. Occasional generalized tonic and clonic contractions of the skeletal muscles. March 10. Temperature 101.0°; paralysis of hind legs; retention of urine. March 11. Paralysis; retention of urine; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous; vessels distended with blood. No evidence of a purulent meningitis. Brain fixed in Zenker's fluid. Sections stained with eosin-methylene blue and according to Giemsa's method.

Section through Hippocampal Region.—Slight general thickening of pia-arachnoid with cellular infiltration consisting of many endothelial leucocytes, a few lymphocytes, and rare polymorphonuclear cells. Occasional acidophilic nuclear inclusion (Fig. 7) in endothelial leucocytes. In places, fixed endothelial cells are prominent and rarely contain inclusions; few mitotic figures; slight perivascular infiltration of penetrating vessels. Generalized meningeal hyperemia with a moderate amount of hemorrhage. Few nuclear inclusions in cells that probably are arachnoidal fibroblasts. Hemorrhage in wall of third ventricle, accompanied by very slight reactive changes. Nuclear inclusions in cells of ependyma (Fig. 6) and choroid plexus. In foci in hippocampus are numerous typical nuclear inclusions; the cellular degeneration is associated with very little, if any, reaction.

Section through Cerebellum.—Meningeal lesions similar to those described above; marked cortical hemorrhage. Nuclear inclusions in outer cells of molecular layer. Pycnotic and fragmentary degeneration of nerve cells of granular layer (Fig. 5). Marked hyaline necrosis of Purkinje cells with pycnosis and chromatolysis of nuclei (Fig. 1). No inclusions found in Purkinje cells.

Rabbit B 6.

March 6. Inoculation similar to that of Rabbit B 5. March 7. Temperature 104°; an'mal appears normal. March 8. Temperature 105°; animal appears normal. March 9. Temperature 102°; sick and irritable. March 10. Paralysis of hind legs. March 11. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. March 12. Paralysis; generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed. Brain edematous. Aerobic and anaerobic cultures showed no growth. Part of brain used for passage of virus, remainder fixed in Zenker's fluid for histological study.

Section through Hippocampal Region.—Diffuse meningitis; some edema, hemorrhage, and fibrin. Nearly all of the cells are endothelial leucocytes; occasional typical nuclear inclusion. Nothing of importance observed in hippocampus.

Section through Cerebellum.—Meningeal reaction similar to that described above.

Some diffuse and marked focal degeneration of Purkinje cells as indicated by the striking oxyphilic reaction involving both the nucleus and the cytoplasm; chromatolysis, karyorrhexis, and a fading out of the Purkinje cells together with adjacent cells of the molecular layer. Throughout the degenerating areas the lack of a significant degree of inflammatory response is noteworthy.

Rabbit B 7.

March 12.   Inoculated intracerebrally with 0.2 cc. of fresh brain Virus III from Rabbit B 6.

March 13.   Temperature 102.8°; animal appears normal. March 14.   Temperature 103.4°; animal appears normal. March 15.   Temperature 104.0°; sick. March 16.   Temperature 104.2°; sick. March 17.   Temperature 104.5°; sick. March 18.   Sick. March 19.   Temperature 101.2°; sick; head pulled to left and rotated. March 20.   Temperature 100.5°; ataxia; loss of weight; head pulled to left; circling. March 21.   Temperature 100.0°; retention of urine. March 22.   Animal prostrate. March 23.   Began to improve and eventually recovered.

Rabbit B 8.

March 12.   Inoculation similar to that of Rabbit B 7.

March 13.   Temperature 102.8°; animal appears normal. March 14.   Temperature 102.4°; animal appears normal. March 15.   Temperature 105.8°; sick; irritable; head retracted. March 16.   Temperature 104.0°; condition worse. March 17.   Temperature 105.0°; condition same. Animal sacrificed for passage of the virus and for histological studies. Cultures of the brain showed no growth.

Sections through the Point of Inoculation and also through the Hippocampus.— Diffuse meningitis—the cells chiefly endothelial leucocytes; a slight generalized invasion of peripheral cortical tissue both over the surface of the brain and also beneath the ependyma. Some perivascular thickening due to endothelial cells. Numerous characteristic nuclear inclusions in endothelial leucocytes and some in arachnoidal fibroblasts, especially in those applied along large vessels. Hyaline degeneration of scattered nerve cells in periphery of cortex. About the site of inoculation, hemorrhage, spongy degeneration of the parenchyma, and a confused cellular picture are observed. All cells are swollen; nuclei are reduced to peripheral chromatin rings with central inclusions. No other structure is sufficiently preserved to enable absolute identification of specific cell type, but it appears that all structures contain inclusions—endothelial, nerve, and glia cells. Marked degeneration of hippocampal cells; swollen nuclei; numerous typical nuclear inclusions.

Rabbit B 29.

April 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 26.

April 20. Temperature 104.8°; animal wild. April 21. Temperature 104.0°; animal wild. April 22. Temperature 105.4°; animal wild. April 23. Temperature 104.5°; tremor. April 24. Temperature 103.0°; marked tremor and ataxia; circling to left; head pulled to left; salivation; twitching of muscles around mouth and of fore legs; occasional generalized tonic and clonic contractions of the skeletal muscles. Animal sacrificed for histological studies. Brain perfused with Zenker's fluid.

The usual meningitis with more polymorphonuclear cells than usually seen. Isolated Purkinje cells show typical hyaline necrosis and nuclear chromatolysis. Nuclear inclusions in ependymal cells lining fourth ventricle and in neighboring glia cells.

Very severe degenerative changes in hippocampus (Fig. 4) with nuclear inclusions in almost every cell (Fig. 2) and extensive spongy degeneration in fiber layers. Slight perivascular hemorrhage. Perivascular infiltration (Fig. 8) consisting of polymorphonuclear cells, lymphocytes, and endothelial leucocytes.

Spongy degeneration and necrosis of nerve cells with typical nuclear inclusion in thalamic region. Epithelial cells of choroid plexus contain nuclear inclusions.

Meningitis with some of the cells showing nuclear inclusions extends to the cervical cord. Slight involvement of periphery of cord and of perivascular sheaths.

Rabbit B 35.

May 19. Inoculated intracerebrally with 0.2 cc. of fresh testicular Virus III from Rabbit T 31.

May 20. Temperature 102.8°; animal seems normal. May 21. Temperature 104.9°; animal seems normal. May 22. Temperature 104.0°; sick. May 23. Temperature 104.0°; worse; tremor. Tremor persisted until May 26, when the rabbit was sacrificed. The brain was perfused with Zenker's fluid. This animal showed no signs of encephalitis except fever, tremor, and a tendency to stand rigidly in one position for long periods of time.

Meninges show lymphocytic, endothelial and plasma cell infiltration, fibrin, and hemorrhage. Nuclear inclusions in endothelial leucocytes and fixed endothelium. Perivascular lymphocytic infiltration along penetrating vessels.

Hyaline necrosis with nuclear karyorrhexis of many Purkinje cells. Nuclear inclusions in small nerve cells interspersed between Purkinje cells, in glia cells, and in endothelial leucocytes. Spongy degeneration of associated fiber layer.

Extensive spongy degeneration with nuclear inclusions in nearly every cell in the hippocampus. Some inclusions in peripheral cortical glia cells and in invading endothelial leucocytes.

A few typical inclusions in glia cells in periphery of cervical cord. Slight endothelial cell infiltration.

In addition to Virus III encephalitis this brain also showed lesions of the spontaneous encephalitis described by Wright and Craighead (7).

From the results of the experiments described above it is obvious that the active agent used is capable of producing an encephalitis in rabbits. The question naturally arises, however, as to whether the virus now under investigation is the one originally encountered 5 years ago, or whether it has become contaminated by another virus, *e.g.*, vaccine virus or herpetic virus.

Relation of Present Virus III to Original Virus III.

Repeated experimental passages of the virus in animals that are occasionally spontaneously infected make it impossible to say definitely that the original strain of Virus III has not been contaminated by a new strain of the active agent. A fortunate circumstance, however, enabled us to demonstrate that the present Virus III is identical with, or at least similar to, the original Virus III. In 1925 Rivers and Pearce (8) found that the transplantable rabbit neoplasm described by Pearce and Brown (9) is infected with Virus III and that the virus persists in the tumor and is regularly passed from rabbit to rabbit with each successive transfer of the tumor. In view of these facts, in order to establish a relationship between the present Virus III and the Virus III of 1925 it was only necessary to determine whether the tumor rabbits are refractory to the active agent now being used. For this purpose Dr. Pearce supplied 6 rabbits that had shown good growths of the tumor.

6 tumor rabbits were inoculated intradermally and intracerebrally respectively with 0.2 cc. of a fresh testicular emulsion containing virus of the same generation as Rabbit B 6 (Text-fig. 1). As controls, an animal which had recovered from encephalitis caused by our virus was inoculated in a similar manner, and 2 normal stock animals received intradermal and intratesticular inoculations. The results showed that the recovered animal and the 6 tumor rabbits had no reaction in the skin and evidenced no signs of encephalitis, while the normal animals had a very marked reaction in the skin and a high fever.

From the results of the above experiment one is justified in concluding that in all probability the virus now being used is identical with the original Virus III.

Consideration of Possible Contaminants.

The next question to arise dealt with the possibility that the emulsions containing Virus III were contaminated by the virus of vaccinia, rabies, or herpes.

Vaccine Virus.—One can be quite positive that the emulsions containing Virus III do not also contain vaccine virus, inasmuch as Dr. Pearce's tumor rabbits, not immune to vaccine virus, are completely protected against the activity of our emulsions. Furthermore, no Guarnieri bodies were observed in cells injured by our active agent.

Rabic Virus.—In making repeated passages in rabbits, it is not inconceivable that one might rarely encounter the virus of rabies. This possibility is very remote. Moreover, Dr. Pearce's tumor animals are immune to our virus, even when inoculated intracerebrally. If rabic virus were a contaminant, her animals, although immune to Virus III, would die of rabies. This did not occur, nor were Negri bodies found in the brains of rabbits dying of Virus III encephalitis.

Herpetic Virus.—The clinical and pathological picture presented by our animals at times so closely resembles that caused by herpetic virus that one naturally would like to know whether our emulsions are contaminated by the virus of herpes.

5 rabbits were chosen; 1 was a normal stock animal, the other 4 had recovered, 4 to 8 weeks previously, from Virus III encephalitis. Each animal was inoculated intracerebrally with 0.2 cc. of a brain emulsion containing H. F. herpetic virus. All of the rabbits showed the usual signs of herpetic encephalitis and were dead within 7 days.

The above experiment clearly indicates that our virus is not contaminated by herpetic virus.

DISCUSSION.

Virus III is an active, filterable agent indigenous to rabbits (2, 5). It undoubtedly causes a natural infection in these animals, yet the spontaneous disease has not as yet been recognized. As previously shown (1–3), the virus under experimental conditions produces a high fever and characteristic lesions in' the cornea, testicles, and skin. Furthermore, within epithelial and endothelial cells of these lesions

acidophilic nuclear inclusions, similar to those seen in varicella and herpes, occur (3).

The studies described in the present paper clearly indicate that Virus III at times is capable of inducing in rabbits an encephalitis which clinically and pathologically closely resembles that caused by herpetic virus. The most interesting fact disclosed by the present work, however, is that the ability of the virus to produce visible evidences of encephalitis seems to vary greatly from time to time (Text-figs. 1 to 3). No adequate explanation of this striking feature is now available. The question as to whether the frequent passages of the virus under experimental conditions, with an occasional freezing and desiccation or storage in glycerol, have altered its activity cannot be answered at present.

The histopathology of experimental Virus III meningoencephalitis in rabbits resembles in part that of herpetic encephalitis. The two diseases are pathologically similar in that both produce a "chronic" type of meningitis, characterized by lymphocytic, plasma cell, and endothelial cell infiltration (Fig. 7); the perivascular sheaths of penetrating vessels may be distended by similar cells (Fig. 8). In both diseases the hippocampal region is profoundly involved; nerve cells, glia cells, and endothelial leucocytes contain characteristic acidophilic nuclear inclusions (Fig. 2); nerve cells undergo hyaline degeneration and seem to disappear rapidly, leaving a spongy, reticulated zone of ground substance (Figs. 2, 4). The adjacent fiber laminæ likewise present a soft, spongy appearance and a few polymorphonuclear and endothelial leucocytes infiltrate the region.

In Virus III encephalitis the next most prominent lesion occurs in the Purkinje cell layer of the cerebellum (Figs. 1, 5). These large cells undergo hyaline necrosis accompanied by nuclear chromatolysis, pycnosis, and karyorrhexis; no inclusions have been observed in their nuclei. Inclusions, however, frequently occur in the smaller nerve cells, in glia cells, and in reacting endothelial leucocytes in the immediate vicinity. The necrosis and disappearance of Purkinje cells leave a zone of spongy degeneration between the granular and molecular layers of the cerebellum. In some animals extensive pycnotic degeneration of nerve cells in the granular layer of the cerebellum (Fig. 5) also occurs. Some of the peculiar clinical manifestations of

the disease are probably due to the cerebellar lesions, but in view of multiple foci of brain involvement one should be cautious in relating the clinical picture to lesions in different anatomical foci.

Meningeal edema and hemorrhage, small hemorrhages at the site of inoculation, and foci of hemorrhage in the deep pontine region further complicate the picture. In some rabbits superficial cortical and subependymal encephalitis was noted; here one finds a spongy degeneration, mitosis of glia and endothelial cells, and infiltration by endothelial leucocytes.

It seems that no type of cell escapes involvement. Inclusions have been seen in nerve cells, glia cells, fixed and mobile endothelial cells, arachnoidal fibroblasts, ependymal cells, and in cells of the choroid plexus. Often one cannot distinguish between a glia cell and an infiltrating endothelial leucocyte because of the loss of characteristic nuclear structure; involved nuclei are reduced to a central, often elongated, inclusion surrounded by a clear zone limited externally by a narrow ring of altered chromatin.

The presence of inclusions, the disappearance of cells and ground substance giving rise to the peculiar soft, spongy, reticular appearance, and the minimal amount of inflammatory reaction agree well with the findings in encephalitis caused by other filterable viruses.

SUMMARY.

Virus III, an active, filterable agent indigenous to rabbits, under experimental conditions produces, in addition to lesions in the cornea, skin, and testicles, an encephalitis which is at times quite similar to that induced by herpetic virus. Virus III and herpetic virus, however, are not immunologically related.

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8. Rivers, T. M., and Pearce, L., Growth and persistence of filterable viruses in a transplantable rabbit neoplasm, *J. Exp. Med.*, 1925, xlii, 523.

9. Pearce, L., and Brown, W. H., Studies based on a malignant tumor of the rabbit. II. Primary transplantation and elimination of a coexisting syphilitic infection, *J. Exp. Med.*, 1923, xxxvii, 631.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Cerebellum. Hyaline necrosis of Purkinje cells. Zenker; eosin-methylene blue. × 500.

FIG. 2. Hippocampus. Cellular degeneration; abundant inclusion bodies in nuclei. Zenker; eosin-methylene blue. × 1000.

FIG. 3. Site of inoculation. Large granular nuclear inclusion in nerve cell. Zenker; eosin-methylene blue. × 1500.

PLATE 13.

FIG. 4. Necrosis of hippocampal cells; spongy degeneration of fiber layers; hyperemia and slight cellular reaction. Zenker; eosin-methylene blue. × 115.

FIG. 5. Pycnotic degeneration of nerve cells of granular layer of cerebellum. Zenker; eosin-methylene blue. × 210.

PLATE 14.

FIG. 6. Inclusions in ependymal cells lining third ventricle. Zenker; eosin-methylene blue. × 1000.

FIG. 7. Endothelial reaction in cortical meninges. Three nuclear inclusions, *a, b, c.* Zenker; Giemsa. × 850.

FIG. 8. Cortex showing perivascular infiltration. Zenker; eosin-methylene blue. × 130.
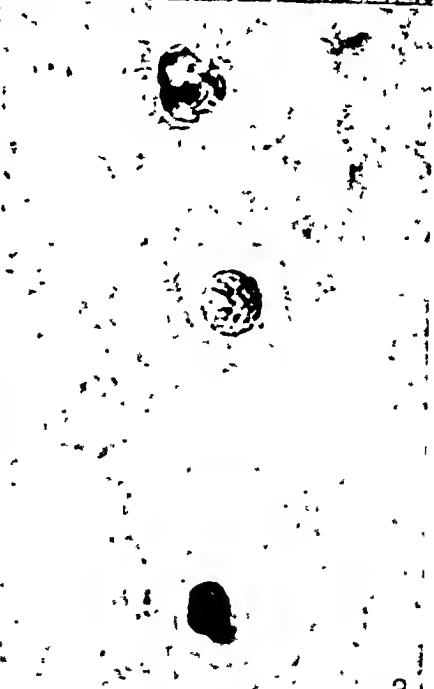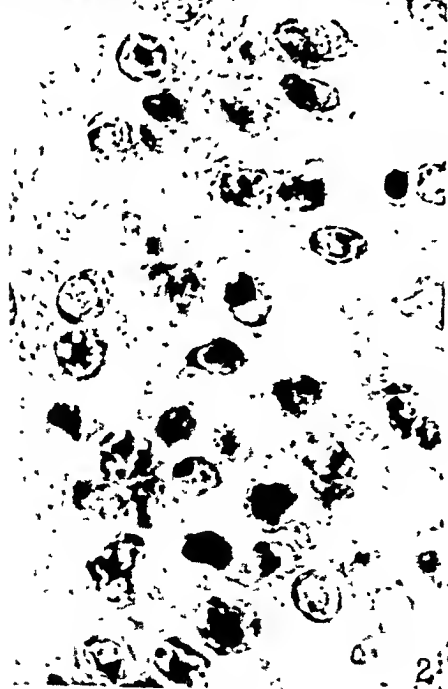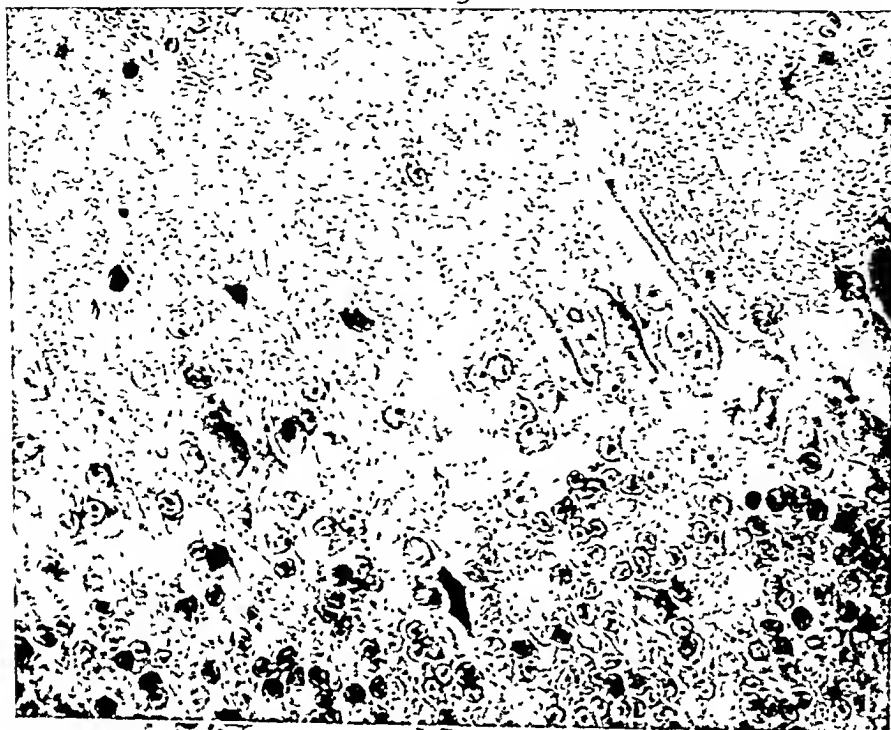
(Rivers and Stewart. Virus III encephalitis.)

(Rivers and Stewart. Virus III encephalitis.)

PLATE 14.
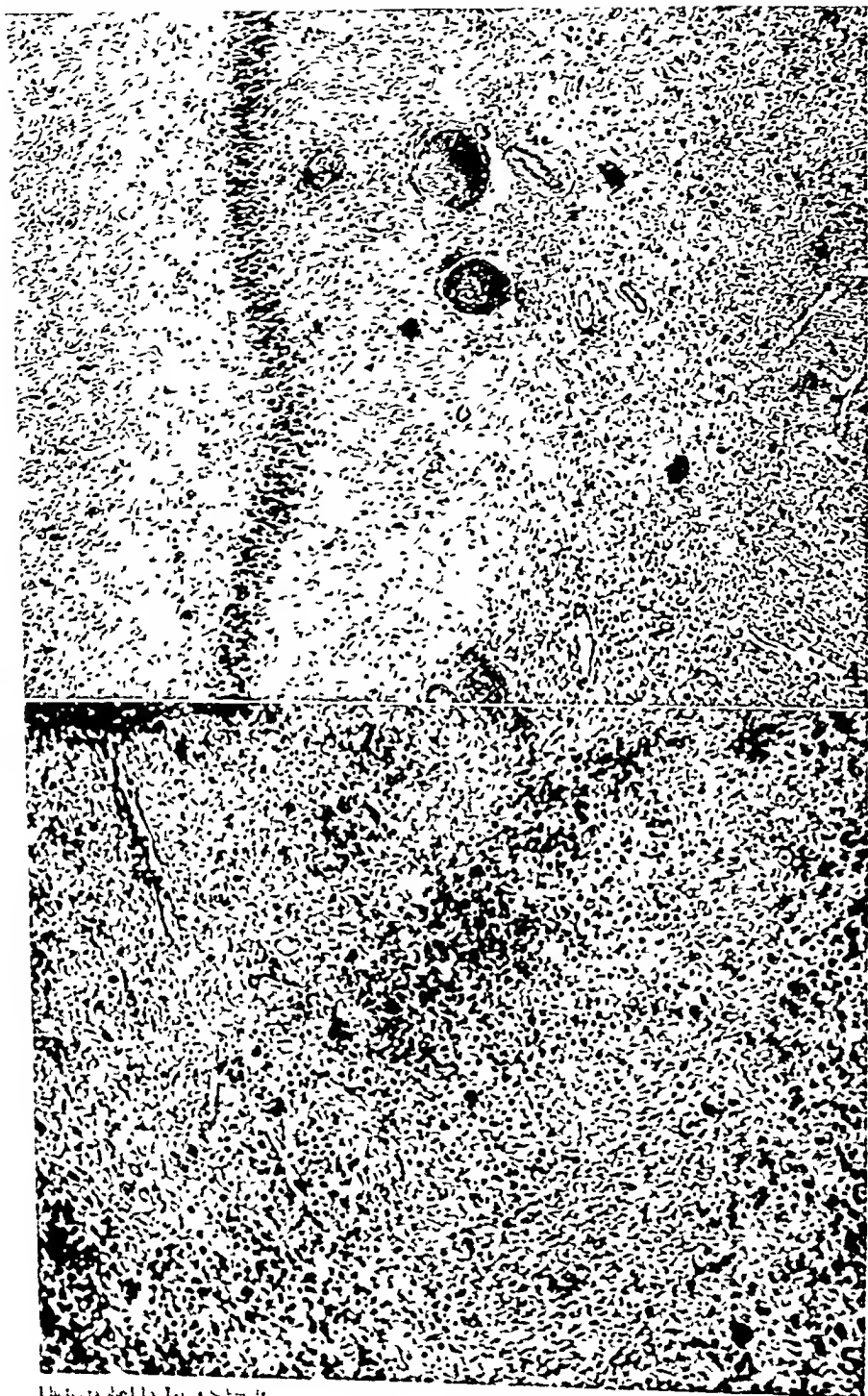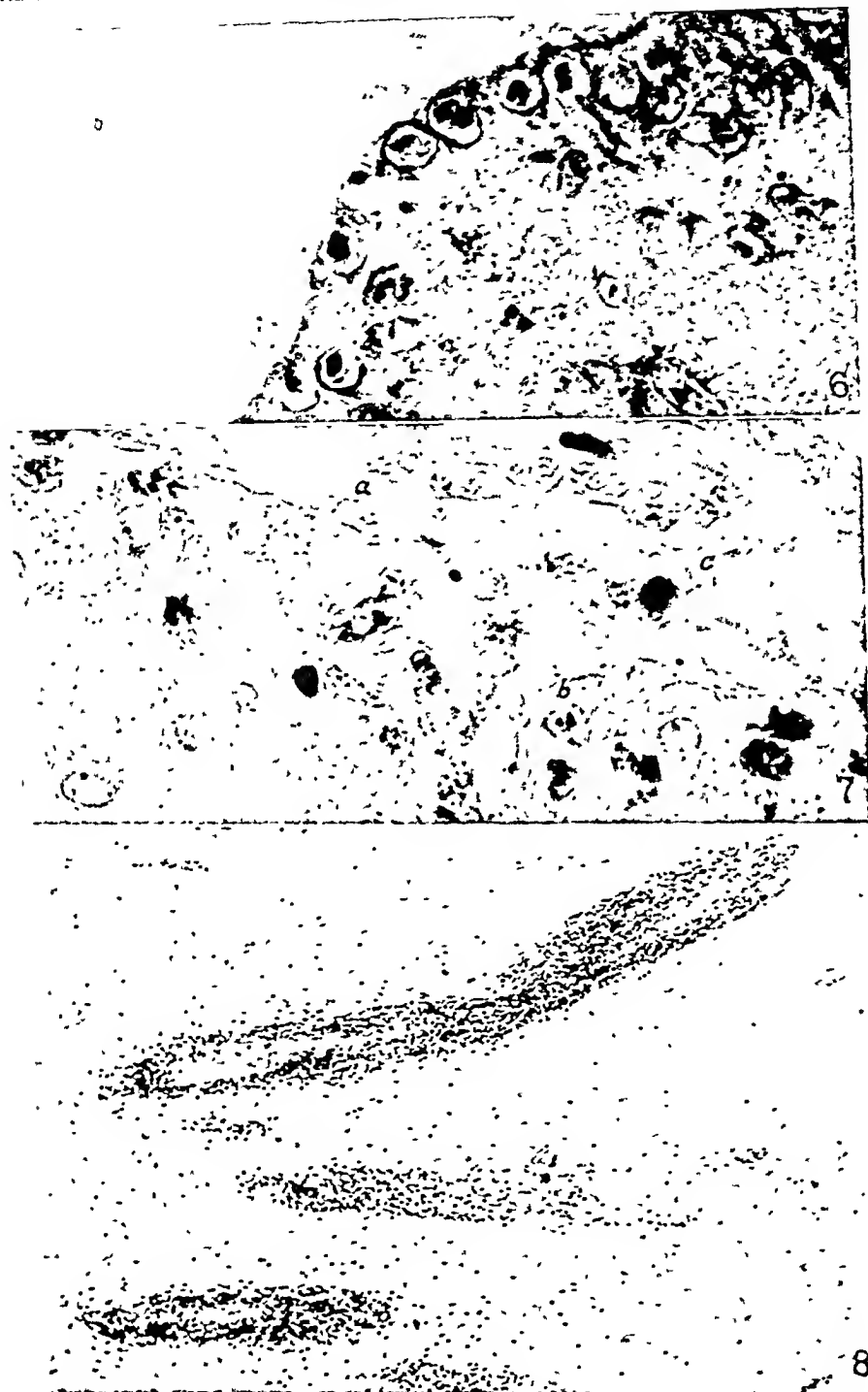
(Rivers and Scott: Virus III encephalitis.)

SURFACE TENSION OF SERA AS AFFECTED BY THE PRECIPITIN REACTION.

By SUSAN GRIFFITH RAMSDELL.

(*From the Research Institute of Cutaneous Medicine, Philadelphia.*)

In a study of the surface tension changes in the blood of the guinea pig in anaphylactic shock, the author concluded[1] that the first lowering of the surface tension was probably due to the simple additive effect of the antigen serum, and that the significant changes were probably due to the effect of secondary tissue products such as cholesterol. But, if the basic reaction in anaphylaxis is a formation of a precipitate in the antigen serum by the cell-combined or circulating antibody, and if the essential effect upon the antigen serum is a denaturing one,—such as occurs in serum following heating, ageing, and shaking, by which the serum loses its capacity to lower the surface tension of a solution in saline,[2]—then one can explain this failure of the anaphylactic serum to show this characteristic change either by the assumption that precipitation plays no part in the phenomenon of shock, or that the guinea pig's serum is too weak in precipitin to afford an appreciable effect on surface tension, or that specific serum precipitation does not affect surface tension in the way that might be expected from that found for other precipitating or flocculating processes. For a study of this last assumption, a precipitating serum of high titre was chosen as furnishing the optimal conditions for any surface tension effect upon either the antibody or the antigen serum as a consequence of precipitation.

The sera of a normal rabbit and of a rabbit immunized against horse serum were studied for such surface tension changes as might occur as a consequence of the precipitin reaction. As antigen, a specially collected lot of normal horse serum was used. In making the readings, the serum in question was finally diluted to 1/10,000 for conformity

[1] Ramsdell, S. G., *J. Exp. Med.*, 1928, xlvii, 993.

[2] du Nouy, P. L., *J. Exp. Med.*, 1922, xxxv, 575.

with previously reported immune serum values.[1,3] The du Noüy tensiometer was used for making the readings.

EXPERIMENTS.

I. *Effect of the Precipitin Reaction upon the Surface Tension of the Immune Serum.*

A mixture was made of 0.2 cc. of an immune rabbit serum, with a precipitin titre of 1/10,000, and 0.8 cc. of a 1/100 dilution of normal horse serum; this was incubated for 1 hour at 38°C., after which a final dilution of the mixture was made so that the resulting rabbit serum dilution was 1/10,000. Static surface tension readings showed a higher value by 2 dynes in this mixture of immune and antigen sera over that shown by the same dilution of the immune serum alone. When a control mixture of normal rabbit serum and antigen, and a dilution of normal rabbit serum alone were read for static surface tension values, there was a change of less than a dyne caused by the presence of the horse serum, and the difference between the static readings of the normal and immune serum mixtures was less than 2 dynes—a difference not significant of any effect of the antigen serum upon the antibody serum.

II. *Effect of Various Dilutions of Antigen Serum upon the Static Reading of the Immune Serum.*

Dilutions of horse serum from 1/10 to 1/100,000 were incubated in the water bath with a fixed amount of undiluted immune serum, in proportion of 0.9 cc. of the former to 0.1 cc. of the latter, for 30 minutes. A definite clouding was evident in the 1/10,000 tube. Dilutions were then made so that the immune serum was in a constant dilution of 1/10,000. There was a regular decrease of the static surface tension value with the increase of concentration of the horse serum from 1/100,-000,000 to 1/10,000, but with only a difference of 3.5 dynes between the highest and the lowest, a value that might be attributed to a simple additive lowering effect of the horse serum.

This conclusion was confirmed by the control experiment where like mixtures and dilutions of normal rabbit serum and horse serum gave a difference of 2.1 dynes between the highest and lowest dilutions of the latter serum.

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[3] du Noüy, P. L., *J. Exp. Med.*, 1922, xxxv, 707.

III. Effect of Precipitin on the Surface Tension of Antigen Serum.

Normal and immune sera, 0.1 cc., were incubated about 20 minutes at 40–50°C. with 1 cc. horse serum, 1/1,000. The immune serum gave a heavy turbidity. Both mixtures were then further diluted to a concentration of horse serum 1/10,000. The difference between the static readings was well within the limits of experimental error. When the experiment was repeated with the horse serum in a dilution of 1/100, which was that giving the maximum precipitate, the results gave even closer values.

Since the relatively great concentration of rabbit serum alone might constitute a factor for the lowering of surface tension in a mixture with antigen, advantage was taken of the high titre (1/40,000), which the immune serum now showed, to use a dilution of 1/20 of the latter in repeating the previous experiment.

The result was the same: the antigen showed no surface tension change that could be ascribed to a denaturing effect by the homologous immune serum.

IV. Effect of the Antigen-Antibody Reaction on Surface Tension in Function of Time.

The last part of Experiment III was repeated, with the difference that the sera were added directly to the horse serum dilution in two series of watch-glasses, and readings were taken at intervals: the resulting curves showed no significant difference in character.

CONCLUSIONS.

An attempt to find evidence, through the study of surface tension, using the du Noüy tensiometer, of a denaturing effect of the precipitin reaction upon either the antigen or the immune serum yielded entirely negative results.

ETIOLOGY OF OROYA FEVER.

XIII. Chemotherapy in Experimental Bartonella bacilliformis Infection.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 15 and 16.

In the course of our studies on *Bartonella bacilliformis* infection in monkeys, we submitted several *Macacus rhesus*, in which experimental verrucous lesions had been induced by means of cultures, to treatment with chemicals which had proved therapeutically useful in spirochetal and leishmania infections. Salvarsan had already been recommended by Arce[1] in the treatment of malignant verruga in man. In our experiments salvarsan, neosalvarsan, bismuth lactate, esters of chaulmoogra oil, sodium gynocardate, neutroflavine, and urotropin were tested. It was not deemed suitable to test tartar emetic, since it is a drug of slow therapeutic action, and the experimental verrucous lesions in the monkey lead in any case to spontaneous retrogression.

Chemical Action in Vitro.

Although it was not expected that a relationship would be shown to exist between the action of the chemical when tested on *Bartonella in vitro* and the verrucous lesions *in vivo*, it was considered of interest to determine the direct effects of the chemicals on the bacilli. The substances to be tested were added directly to the culture media, and the cultures were incubated at 25°C. for a period of 13 days. The results are shown in Table I. Neutroflavine inhibited growth in 1:10,000,000 dilution. Formalin was almost as effective, and neosalvarsan, novasurol, and mercuric chloride were effective up to

[1] Arce, J., *An. Facultad Med. Lima*, 1918, i, No. 3, 21–53, 130–161; No. 4, 24–52.

TABLE I.

Growth-Inhibiting Properties in Vitro.

	Final concentration of substance in culture medium					
	1:100	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Bismuth albuminate	− (Turbid)	− (Turbid)	++++	++++	++++	++++
Tartar emetic	− (Turbid)	− (Turbid)	−	+	++++	++++
Neosalvarsan	± (Clear, deep brown)	− (Brown)	− (Yellowish)	−	++	++++
Tryparsamide	(Clear)	++++	++++	++++	++++	++++
Mercuric chloride	− (Turbid)	− (Clear)	−	−	++++	++++
Novasurol	− (Clear)	−	−	−	++++	++++
Mercurochrome	− (Deep red, clear)	− (Deep red)	− (Eosin red)	++++ (Lt. eosin)	++++ (Tr. pink)	++++
Neutroflavine	(Turbid, deep gold)	− (Turbid, gold)	− (Greenish yellow, clear)	− (Greenish)	− (Lt. green)	− (Tr. green)
Optochin	(Turbid, white)	− (Opalescent)	+ (Clear)	+++	+++	+++
Sodium taurocholate	(Turbid, brownish)	?	+++ (Clear)	+	+++	+++
Phenol	(Opalescent)	?		+++	+++	+++
Formalin	− (Clear)	−	−	−	±	+++
Lugol's solution	− (Clear)	++++	++++	++++	++++	++++

− = complete inhibition of growth.

++++ = no inhibition of growth.

1:100,000. Mercurochrome and tartar emetic required at least 1:10,000 concentration to prevent growth.

The first experiments were made on monkeys in which the cherry-red verrucous lesions on the abdominal skin and eyebrows had reached maximal size and had persisted in this state for several days. Blood cultures taken shortly before or at the time of first injection of the chemical into the circulation proved subsequently to be negative, and bits of excised nodules taken at the same time[2] showed few bacilli or even none at all by culture or in section. These last findings could not be known at the time of treatment, since the bacilli require 10 to 14 days to become evident in culture.

However, distinction between the ordinary or spontaneous regression of the nodules and the regression taking place after the use of chemicals, is entirely possible. The mature nodules undergo spontaneous regression slowly,[3] while in the animals given chemicals there occurred rapid loss of cherry-red color, usually in 24 to 48 hours after the first injection, followed by a still more rapid reduction in size most pronounced in the nodules located in the abdominal skin. At the expiration of 5 to 6 days small pale fibrous areas alone remained to indicate the site of the nodules, and in 10 to 14 days all vestiges had disappeared, the lesions of the eyebrows persisting somewhat longer than those of the abdomen. The protocols of these experiments follow.

Protocols.

Macacus rhesus 1-T, injected intravenously on Oct. 14, 1926, with 0.5 cc. of a mixture of:

4 cc. defibrinated blood (culture $+ + +$) from *M. rhesus* 54 (P. 5 strain[4]).

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown on leptospira medium for 72 hours,

5 cc. culture of *Bartonella bacilliformis* (P. 5 strain) grown for 6 days on blood agar slants.

In addition, 4 intradermal injections of the mixture were made on the left abdominal wall and 2 on the left eyebrow. Also nodular tissue freshly excised from *M. rhesus* 54 was applied to scarified areas on the right abdominal wall and right eyebrow.

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[2] Ether anesthesia was used in all the operations.

[3] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 455.

[4] Noguchi, H., *J. Exp. Med.*, 1927, xlv, 175.

Experimental nodules appeared on the left eyebrow in 14 days. Within a month large nodules were present on the abdomen at the sites of intradermal inoculation, and the scarified areas showed the characteristic miliary lesions. On Nov. 23 (40 days after inoculation) the hemoglobin was 35 per cent (Sahli), and the red cells 2,502,000, and there had been no fever. 0.05 gm. of salvarsan was given intravenously. Within the following week the nodules grew small and became paler. On Dec. 3 a second injection of 0.05 cc. salvarsan was administered. Within the week the nodules had become very small and pale, the erythrocytes rose to 5,600,000 and the hemoglobin to 80 per cent. When the animal was sacrificed on Dec. 13, *Bartonella bacilliformis* could not be detected in the nodular tissue either microscopically or by culture, and blood, lymph nodes, and spleen also failed to yield cultures. Sections of the nodules showed fibrous tissue.

Macacus rhesus 2-T. Inoculated at the same time as No. 1-T, and in the same manner, except that the eyebrows were spared. On Oct. 19 the culture titer of the blood was 1:100,000. On Nov. 15, or 32 days after the inoculation, the animal showed large mature subcutaneous nodules on the left abdominal wall and numerous red miliary lesions on the scarified areas on the right side (Fig. 1). Blood cultures made on this day later proved to be negative. A mixture of 1 cc. of 1 per cent bismuth lactate, 1 cc. of 1 per cent neutroflavine, and 1 cc. of 1 per cent urotropin was injected intravenously. The temperature rose to 105.2°F. on the following day (Nov. 16), but the animal appeared well. On Nov. 23, a second injection of the same mixture was given. The nodules soon became bluish, smaller in size, and continued to diminish in volume after the second dose of the drugs (Fig. 2). On Dec. 3 a third injection was given. During the following 9 days the nodules became very small and pale (Fig. 3). Nodules and spleen removed on Dec. 16 did not yield *Bartonella bacilliformis* in culture.

Macacus rhesus 3-T, inoculated in same manner as *Macacus* 2-T. By Nov. 24 the abdomen showed large, mature subcutaneous nodules (Fig. 4), and large bluish red nodules on each leg where the injections had been made into the saphenous veins. A general miliary eruption was also present on the abdomen (Fig. 5). The hemoglobin was 45 per cent (Sahli), and the red blood cells 3,960,000. No fever. A suspension of one of the nodules yielded cultures of *Bartonella bacilliformis* in a dilution of 1:1,000, but the blood proved negative, although the titer had been 1:100,000 on Oct. 19 and Nov. 3. At this time (*i.e.*, on Nov. 24, 41 days after inoculation), 1 cc. of moogrol[5] was given intravenously. Within 48 hours the nodules had become bluish in color and somewhat smaller. Fig. 6 shows the appearance of the nodules 9 days after the first treatment. On Dec. 3 another injection of 1 cc. of moogrol was given. The temperature rose on the 2 days following to 104.4–104.6°F. By Dec. 13 the nodules had contracted considerably (Fig. 7). The spleen was negative for culture on Dec. 16, and all lesions had disappeared by Dec. 30. The blood culture was negative a week later.

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[5] Burroughs Wellcome and Company's preparation of the esters of chaulmoogra oil.

Macacus rhesus 4-T was inoculated intravenously on Nov. 24, 1926, with 1 cc. of a mixture of cultures of *Bartonella bacilliformis,* besides which a suspension of an abdominal nodule of *M. rhesus* 3-T was given intravenously and introduced intradermally into both eyebrows and the abdominal skin. On Dec. 21 the blood cultures were positive for *Bartonella bacilliformis* in a 1:100,000 dilution. The nodules were fully developed by Dec. 28, 34 days after inoculation (Figs. 8 and 11), when the first intravenous injection of bismuth lactate, proflavine, and urotropin was given (a mixture of 1 cc. of a 1 per cent solution of each). Blood cultures were negative at this time. The temperature rose to 105°F. on the day following the treatment, and the nodules had already shrunk and become paler. A double dose was given on Dec. 30, and again on Jan. 3, 1927. No febrile reactions. Blood culture was negative on Jan. 5, 1927. The hemoglobin was 78 per cent (Sahli), and the erythrocytes 4,400,000. Cultures made from nodules and lymph nodes on Jan. 10, 1927, were negative. The lesions regressed rapidly (Figs. 9 and 12), and only fibrous traces remained on Jan. 28, 1927 (Figs. 10 and 13).

Macacus cynomolgus 5-T, inoculated intradermally with a suspension of nodular tissue from *M. rhesus* 1-S[6] into the right eyebrow and the abdominal skin on Dec. 7, 1926. This animal remained afebrile but developed large cherry-red nodules on eyebrows and abdomen by Dec. 27. The blood culture titer was 1:10 on Jan. 6, 1927. On Jan. 8 and 14, 1927, or 32 and 38 days after inoculation, an intravenous injection was made of 1 cc. of 5 per cent bismuth lactate, 1 cc. of 5 per cent urotropin, and 1 cc. of 1 per cent neutroflavine. Figs. 14 and 15 show the appearance of the lesions on Jan. 7. The nodules began to shrink in the following week and within 2 weeks had become small and fibrous. The appearance on Feb. 8 is shown in Figs. 16 and 17.

The next step was to test the action of the chemicals on the appearance and development of the nodules in instances in which the chemicals were administered *before* the lesions reached full development, *i.e.,* 2 to 3 weeks after the inoculation of the infective material into the skin, while the lesions were growing in size daily. Under these circumstances the drugs failed to influence the progress of the lesions, which in one instance attained unusually large proportions (Figs. 19, 20). The usual cherry-red color developed without hindrance, and the scarified areas became covered with the characteristic deep red miliary nodules. As in untreated animals, the skin of the lower half of the abdomen became edematous.

[6] Noguchi, H., *J. Exp. Med.,* 1928, xlvii, 821.

Protocols.

Macacus rhesus 6-T, inoculated intravenously (2 cc.) and intradermally on Dec. 28, 1926, with a mixture of cultures of *Bartonella bacilliformis* and a suspension of nodular tissue of *M. rhesus* 4-T. The nodules were well advanced on Jan. 24, 1927, 27 days after the inoculation, when intravenous injection of a mixture of bismuth, urotropin, and proflavine (1 cc. of 1 per cent proflavine, 1 cc. of 5 per cent urotropin, and 1 cc. of 5 per cent bismuth lactate) was begun. Blood taken just before the treatment yielded cultures in a dilution of 1:10. No change was observed in the nodules after the first injection. The second injection, given on Jan. 26, 1927, was followed by slight diminution in the size of the nodules on the eyebrows while the abdominal lesions continued to enlarge.

Macacus rhesus 7-T, inoculated by scarification and intradermal injection on Mar. 8, 1927, with a suspension of the nodule from *M. rhesus* 3-A, which had been infected by means of the L_5 strain of *Bartonella bacilliformis*.[7] Small nodules were present 20 days after inoculation (Fig. 18), when the animal was given the first intravenous injection of 0.1 gm. of neosalvarsan. The injection was repeated 2 days later. The nodules continued to grow gradually and within 2 weeks they attained unusually large dimensions (Figs. 19 and 20). Certainly no inhibitory action was apparent.

Macacus rhesus 8-T. This animal was inoculated in the same manner and on the same date as the foregoing monkey. Nodules had appeared by Mar. 28 (Fig. 21), when an intravenous injection of 2 cc. of 3 per cent sodium gynocardate and an intramuscular injection of 0.15 cc. of chaulmestrol[8] were given. This was followed 2 days later by an injection of 3 cc. of the sodium gynocardate and an injection of 0.2 cc. of chaulmestrol. No inhibitory effect upon the development of the cutaneous lesions was apparent and the large nodules are shown in Figs. 22 and 23.

SUMMARY.

The therapeutic effect of several antiparasitic chemicals on experimental verruga peruana is described. The drugs were administered by intravenous injection according as the nodules (1) were already developed to an approximate maximum, or (2) were still in the active period of growth.

The effect of the drugs was different under the two circumstances of

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[7] Noguchi, H., *J. Exp. Med.*, 1928, xlvii, 219.

[8] The name given by the Winthrop Chemical Company to their preparation of the esters of chaulmoogra oil, of which the Company kindly furnished a sample.

their administration. When they were given after the maturity of the nodules they hastened the regressive process, but when given during active growth of the lesions no action whatever was detected.

Bartonella bacilliformis in culture is acted upon injuriously by a number of the chemicals employed in the therapeutic tests, the most active being formalin and neutroflavine.

THE EFFECT OF SODIUM CHLORIDE ON THE CHEMICAL CHANGES IN THE BLOOD OF THE DOG AFTER OBSTRUCTION OF THE CARDIAC END OF THE STOMACH.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas, School of Medicine, Kansas City, Kansas.*)

After obstruction of the pyloric end of the stomach, there is a rapid fall in the blood chlorides, an increase in the CO_2 combining power, and a rise in the non-protein nitrogen (1). With the vomiting which necessarily follows pyloric obstruction some chloride is lost in the vomitus. In attempting to eliminate the loss of chloride in this manner, the blood chemistry of the dog was studied after ligation of the cardiac end of the stomach (2). It was found in such experiments, that there is a rapidly developing toxemia with marked rise in non-protein nitrogen and usually some fall in the chlorides. The toxemia is even more marked than that following pyloric and high intestinal obstruction.

It has been shown that solutions of sodium chloride will prevent or relieve the characteristic toxemia of experimental pyloric and intestinal obstruction (3). The experiments reported herewith have been made to determine if solutions of sodium chloride have a similar effect on the toxemia of cardiac obstruction.

Method.

The animals have been kept in metabolism cages during the experiments. No food was given for 24 hours preceding operation and no food or water during the course of the experiments. All ligations were done under ether anesthesia with aseptic technique. Local anesthesia was employed when releasing the obstruction. The obstruction was made by tying tape around the cardiac end of the stomach as closely as possible to the lower end of the esophagus.

The non-protein nitrogen of the blood was determined by the method of Folin and Wu (4), the CO_2 combining power by the method of Van Slyke and Cullen (5),

the urea nitrogen by the Van Slyke and Cullen modification of the Marshall method (6), and the chlorides on the tungstic acid filtrate as suggested by Gettler (7). The nitrogen in the urine was determined by a micro Kjeldahl method (8) and the chlorides by a modified Volhard-Arnold method.

TABLE I.

Effect of Simple Obstruction of Cardiac End of Stomach.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		ms.	*ms.*	*ms.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
4–48	0	33.0	16.1	470	40.0	100	0.27	0.27	0.46	0.46	No treatment
(wt.	1	59.5	30.1	450	40.0	225	0.34	0.77	1.38	3.10	
17 kg.)	2	146.0	87.6	460	30.5	455	0.75	3.41	0.96	4.37	
	3					140	1.00	1.40	0.62	0.87	
4–49	0	27.7	12.6	520	36.2	105	0.10	0.11	0.21	0.22	No treatment
(wt.	1	32.6	11.2	460	30.5	300	1.03	3.10	2.00	6.0	
17 kg.)	2	51.8	27.9	420	32.4	500	0.64	3.20	0.40	2.0	
	3	68.8	28.7	530	38.1	485	0.47	2.28	2.00	9.7	
	4					320	0.50	1.60	1.50	4.80	
4–51	0	28.9	9.8	470	40.0						No treatment
(wt.	1	31.9	11.8	450	43.8	535	1.40	7.44	1.32	7.06	
20 kg.)	2	82.5	46.9	440	34.3	380	0.68	2.59	1.44	5.47	
	3	139.0	84.1	400	32.4	420	0.65	2.73	0.82	3.44	
	4					105	0.48	0.50	0.62	0.65	

OBSERVATIONS.

Table I shows the characteristic findings in blood and urine after obstruction of the cardia. There is a rapid rise in non-protein nitrogen, very little change in the CO_2 combining power, and some tendency toward a fall in chlorides. A marked diuresis occurs with a high nitrogen and chloride output. One animal died before the 3rd day and two before the 4th day.

In Table II are recorded the findings in three dogs similarly obstructed and given 40 cc. per kilo body weight of 1 per cent sodium

TABLE II.

Obstruction of Cardiac End of Stomach.

Treatment with 1 Per Cent Sodium Chloride Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		mg	mg	mg	vol. per cent	cc.	per cent	gm	per cent	gm.	
5–47 (wt. 11 kg.)	0	33 7	17 5	520	52 0	50	0 78	0 39	1.44	0.72	Operation. 40 cc. NaCl per kg. injected
	1	24.0	11.9	480	36.2	250	0 95	2.38	0 62	1.60	40 cc. NaCl per kg. injected
	2	29 0	16.1	580	32 4	870	0 63	5 50	1.04	9.2	" "
	3	23.4	10 5	550	38.1	285	0 77	2 20	0 96	2 8	" "
	4	28 2	16.1	620	47.5	215	0 84	1.80	1.10	2.4	" "
	5	40 0	14.7	600	38.1	205	0 80	1.64	1.32	2.8	" "
	6	30 0	17.5	650	41.9						" "
	7	27 3	15.4	690	40 0	135	1 20	1 62	0 46	0.64	No injection
	8	27 5	18.9	600	45 7	110	1.06	1 17	1 02	11.12	" "
	9	29 4	18 2	670	34 3						Injection given
	10	25 9	14.0	680	41 9						No injection
	11	31.6	20 3	640	34 3	210	1.10	2 31	1 50	3.2	" "
	12	68 3	35 0	640	34 3						Injection given
	13	36 9	20 3	660	38.1	225	0 95	2 14	1 56	3.6	" "
	14					100	0 93	0 93	1 23	1.23	No injection
	15	39 0	25 9	690	32 4	85	1.70	1.45	1 20	1.08	" "
	17	46 5	23 8	780	32.4						" "
	18					170	1 69	2 87	1 10	1.9	Injection given
	19	48 5	21 7	630	30 5						" "
	21					130	0 61	0 79	1 44	1.9	No injection
	22	30 6	20 1	630	28 7						Injection given
	23					190	1 58	3 02	1 84	3 5	No injection
	24	28 9	17 3	610	30 5	79	1 83	1 43	1 68	1 3	Injection given
	26	25 2	15 4	590	25 6	180	1 85	3 33	1 34	2 0	" "
	28	27 0	13 5	620	32 4	65	1 05	0 70	1 41	0 9	" "
	29					100	1 80	1 79	1 41	1 4	No injection
	30	29 1	17 8	540	41 9						Injection given
	31					140	1 70	2 31	1 50	2 1	No injection
	32	26 3	11 2	540	34 3	170	1 69	2 72	1 25	2 1	Injection given
	33					200	1 45	2 90	1 37	2 7	" "

TABLE II—*Continued*.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	Amount	Chlorides		Nitrogen		
		ms.	ms.	ms.	vol. per cent	cc.	per cent	gm.	per cent	gm.	
5–47 (continued)	34	51.0	24.8	540	41.9	215	0.53	1.14	1.72	3.7	No injection
	35					175	0.52	0.91	1.60	2.8	Injection given
	36	58.3	35.0	540	38.1	90	0.35	0.32	1.74	1.6	Died
5–48 (wt. 9.6 kg.)	0	36.1	21.7	450	30.5						Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	24.4	9.8	470	32.4	660	0.61	4.0	0.28	1.9	40 cc. 1 per cent NaCl per kg. injected
	2	33.3	14.0	550	29.6	470	0.72	3.4	0.28	1.3	" "
	3	24.4	12.6	520	32.4	195	0.77	1.5	0.76	1.5	" "
	4	22.8	14.7	570	32.4	155	1.03	1.6	1.00	1.5	" "
	5	32.4	16.4	590	28.7	135	1.21	1.6	1.32	1.9	" "
	6	34.5	25.2	620	30.5						No injection
	7	39.1	25.2	590	29.6						No injection
	8	43.2	32.2	560	29.6	155	1.36	2.1	1.20	1.9	Injection given
	9	45.7	27.9	570	30.5						" "
	10	27.0	17.5	560	30.5						No injection
	11	30.0	16.1	540	27.5						" "
	12	50.5	21.7	640	27.5	110	1.18	1.3	1.44	1.6	" "
	13	44.2	32.9	550	30.5						Injection given
	15	40.0	31.5	550	28.7	150	1.69	2.5	1.32	2.0	No injection
	17	46.3	20.1	530	28.7						" "
	18			540		125	0.79	1.0	1.50	1.9	" "
	19	48.7	27.3	540	28.7						Injection given
	21					80	0.96	0.8	1.64	1.3	No injection
	22	41.7	22.9	540	25.6						Injection given
	23					80	1.50	1.2	1.60	1.3	No injection
	24	42.2	25.7	570	23.0	120	1.61	1.9	1.88	2.3	Injection given
	26	22.9	15.9	550	25.6	130	1.86	2.4	1.64	2.1	" "
	27										No injection
	28	22.2	10.3	550	27.7	155	1.32	2.1	1.07	1.7	Injection given
	29					160	1.34	2.2	1.25	2.0	No injection
	30	26.1	10.7	500	28.7						Injection given
	31					200	1.41	2.8	1.08	2.2	" "
	32	23.0	9.3	490	30.5	130	0.97	1.3	0.93	1.2	" "
	33					110	2.10	2.3	1.25	1.4	" "
	34	20.4	8.9	490	27.7	135	1.27	1.7	0.97	1.3	No injection

TABLE II—*Concluded.*

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides		Nitrogen		
		mg.	mg.	mg.	vol. per cent	cc.	per cent	gm.	per cent	gm.	
5–48 (continued)	35					95	1.28	1.2	0.98	1.0	Injection given
	36	24.2	14.5	500	34.3	90	0.82	0.7	1.56	1.4	" "
	38	22.8	9.8	560	30.5						" "
	40					200	1.16	2.3	1.98	4.0	" "
	43					210	0.88	1.9	2.34	5.0	No injection
	45	24.0	10.3	520	32.4						Died
5–55 (wt. 18 kg.)	0	25.0	15.4	470	32.4	65	1.05	0.68	0.66	0.42	Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	25.2	12.6	470	37.2	165	0.99	1.62	0.96	1.63	40 cc. 1 per cent NaCl per kg. injected
	2	22.5	15.4	520	37.2	225	1.15	2.59	1.04	2.34	" "
	3	25.2	14.0	510	38.1	480	1.50	7.20	0.86	4.13	" "
	4	27.7	10.5	510	38.1	115	1.30	1.50	0.76	0.91	" "
	5	22.5	14.0	540	34.3						" "
	6	26.3	10.5	560	36.2	670	1.34	8.98	0.59	3.95	" "
	7	23.3	8.4	600	36.2	220	1.62	3.56	1.04	2.10	" "
	8					225	1.50	3.38	1.10	2.53	No injection
	9	25.2	14.9	580	31.5						Injection given
	10	21.6	12.6	610	35.3	130	1.92	2.50	2.68	3.50	No injection
	11	30.0	21.0	550	26.8	80	1.91	1.53	2.04	1.63	Injection given
	13	23.6	10.3	560	32.4	100	1.96	1.96	1.88	1.88	" "
	14					190	1.93	3.67	1.94	3.69	No injection
	15	21.8	8.4	530	30.5	110	1.94	2.14	1.72	1.90	Injection given
	16					80	1.96	1.57	1.56	1.25	No injection
	17	30.9	15.9	470	30.5						Injection given
	18					160	1.93	3.09	2.22	3.6	No injection
	19	28.0	15.9	480	35.3	120	1.86	2.23	1.34	1.61	Injection given
	20					440	1.40	6.16	0.72	3.17	" "
	21	75.8	43.4	450	36.2	125	1.04	1.31	0.65	0.81	No injection
	22					130	1.05	1.37	0.77	1.0	Injection given
	23	24.8	9.3	430	38.1	160	1.69	2.71	1.68	2.69	" "
	25	23.4	6.5	440	36.2						" "
	27					320	1.18	3.78	0.73	2.34	" "
	29	26.8	9.3	470	38.1						No injection
	30					180	1.50	2.70	1.45	2.61	
	32	27.0	7.4	450	34.3						Died

TABLE II—*Continued.*

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides		Nitrogen		
		$mg.$	$mg.$	$mg.$	vol. per cent	$cc.$	per cent	$gm.$	per cent	$gm.$	
5–47 (continued)	34	51.0	24.8	540	41.9	215	0.53	1.14	1.72	3.7	No injection
	35					175	0.52	0.91	1.60	2.8	Injection given
	36	58.3	35.0	540	38.1	90	0.35	0.32	1.74	1.6	Died
5–48 (wt. 9.6 kg.)	0	36.1	21.7	450	30.5						Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	24.4	9.8	470	32.4	660	0.61	4.0	0.28	1.9	40 cc. 1 per cent NaCl per kg. injected
	2	33.3	14.0	550	29.6	470	0.72	3.4	0.28	1.3	" "
	3	24.4	12.6	520	32.4	195	0.77	1.5	0.76	1.5	" "
	4	22.8	14.7	570	32.4	155	1.03	1.6	1.00	1.5	" "
	5	32.4	16.4	590	28.7	135	1.21	1.6	1.32	1.9	" "
	6	34.5	25.2	620	30.5						No injection
	7	39.1	25.2	590	29.6						No injection
	8	43.2	32.2	560	29.6	155	1.36	2.1	1.20	1.9	Injection given
	9	45.7	27.9	570	30.5						" "
	10	27.0	17.5	560	30.5						No injection
	11	30.0	16.1	540	27.5						" "
	12	50.5	21.7	640	27.5	110	1.18	1.3	1.44	1.6	" "
	13	44.2	32.9	550	30.5						Injection given
	15	40.0	31.5	550	28.7	150	1.69	2.5	1.32	2.0	No injection
	17	46.3	20.1	530	28.7						" "
	18					125	0.79	1.0	1.50	1.9	" "
	19	48.7	27.3	540	28.7						Injection given
	21					80	0.96	0.8	1.64	1.3	No injection
	22	41.7	22.9	540	25.6						Injection given
	23					80	1.50	1.2	1.60	1.3	No injection
	24	42.2	25.7	570	23.0	120	1.61	1.9	1.88	2.3	Injection given
	26	22.9	15.9	550	25.6	130	1.86	2.4	1.64	2.1	" "
	27										No injection
	28	22.2	10.3	550	27.7	155	1.32	2.1	1.07	1.7	Injection given
	29					160	1.34	2.2	1.25	2.0	No injection
	30	26.1	10.7	500	28.7						Injection given
	31					200	1.41	2.8	1.08	2.2	" "
	32	23.0	9.3	490	30.5	130	0.97	1.3	0.93	1.2	" "
	33					110	2.10	2.3	1.25	1.4	" "
	34	20.4	8.9	490	27.7	135	1.27	1.7	0.97	1.3	No injection

TABLE II—*Concluded*.

Dog No.	Day after operation	Non-protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides per cent	Chlorides gm.	Nitrogen per cent	Nitrogen gm.	Remarks
		mg.	mg.	mg.	vol. per cent	cc.	per cent	gm.	per cent	gm.	
5–48 (continued)	35					95	1.28	1.2	0.98	1.0	Injection given
	36	24.2	14.5	500	34.3	90	0.82	0.7	1.56	1.4	" "
	38	22.8	9.8	560	30.5						" "
	40					200	1.16	2.3	1.98	4.0	" "
	43					210	0.88	1.9	2.34	5.0	No injection
	45	24.0	10.3	520	32.4						Died
5–55 (wt. 18 kg.)	0	25.0	15.4	470	32.4	65	1.05	0.68	0.66	0.42	Operation. 40 cc. 1 per cent NaCl per kg. injected
	1	25.2	12.6	470	37.2	165	0.99	1.62	0.96	1.63	40 cc. 1 per cent NaCl per kg. injected
	2	22.5	15.4	520	37.2	225	1.15	2.59	1.04	2.34	" "
	3	25.2	14.0	510	38.1	480	1.50	7.20	0.86	4.13	" "
	4	27.7	10.5	510	38.1	115	1.30	1.50	0.76	0.91	" "
	5	22.5	14.0	540	34.3						" "
	6	26.3	10.5	560	36.2	670	1.34	8.98	0.59	3.95	" "
	7	23.3	8.4	600	36.2	220	1.62	3.56	1.04	2.10	" "
	8					225	1.50	3.38	1.10	2.53	No injection
	9	25.2	14.9	580	31.5						Injection given
	10	21.6	12.6	610	35.3	130	1.92	2.50	2.68	3.50	No injection
	11	30.0	21.0	550	26.8	80	1.91	1.53	2.04	1.63	Injection given
	13	23.6	10.3	560	32.4	100	1.96	1.96	1.88	1.88	" "
	14					190	1.93	3.67	1.94	3.69	No injection
	15	21.8	8.4	530	30.5	110	1.94	2.14	1.72	1.90	Injection given
	16					80	1.96	1.57	1.56	1.25	No injection
	17	30.9	15.9	470	30.5						Injection given
	18					160	1.93	3.09	2.22	3.6	No injection
	19	28.0	15.9	480	35.3	120	1.86	2.23	1.34	1.61	Injection given
	20					440	1.40	6.16	0.72	3.17	" "
	21	75.8	43.4	450	36.2	125	1.04	1.31	0.65	0.81	No injection
	22					130	1.05	1.37	0.77	1.0	Injection given
	23	24.8	9.3	430	38.1	160	1.69	2.71	1.68	2.69	" "
	25	23.4	6.5	440	36.2						" "
	27					320	1.18	3.78	0.73	2.34	" "
	29	26.8	9.3	470	38.1						No injection
	30					180	1.50	2.70	1.45	2.61	
	32	27.0	7.4	450	34.3						Died

chloride subcutaneously at varying intervals.   There is no rise in
non-protein or urea nitrogen.   The CO_2 combining power shows little

TABLE III.

Obstruction of Cardiac End of Stomach.

Treatment with 2 Per Cent Glucose Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides		Nitrogen		
		mg.	mg.	mg.	vol. per cent	cc.	per cent	gm.	per cent	gm.	
5–60 (wt. 13 kg.)	0	29.4	17.3	420	28.7						Operation. 40 cc. of 2 per cent glucose per kg. injected
	1	33.0	24.3	410	29.6	700	0.88	6.13	1.30	9.1	40 cc. of 2 per cent glucose per kg. injected
	2	123.0	73.8	380	20.2	230	1.15	2.65	0.63	1.5	" "
	3					140	0.70	0.98	1.36	1.9	Died preceding night
5–61 (wt. 15 kg.)	0	34.9	23.4	520	38.1						Operation. 40 cc. 2 per cent glucose per kg. injected
	1	44.4	35.0	420	27.7	800	0.62	4.96	2.08	16.8	40 cc. of 2 per cent glucose per kg. injected
	2	138.0	66.9	400	29.6	400	0.84	3.36	0.47	1.9	" "
	3	188.0	83.6	380	18.3						" "
	4					250	0.53	1.33	0.40	1.0	Died
5–62 (wt. 15.5 kg.)	0	45.6	22.9	460	35.3						Operation. 40 cc. 2 per cent glucose per kg. injected
	1	39.4	21.5	400	25.8	900	1.31	11.8	2.5	22.5	40 cc. of 2 per cent glucose per kg. injected
	2	63.5	29.4	390	29.6	250	1.11	2.8	0.75	1.9	" "
	3	107.0	61.2	360		350	0.45	1.6	2.04	7.1	" "
	4					70	0.33	0.3	1.07	0.8	Dead

change; the chlorides tend to remain higher than normal.   All three
animals show the initial diuresis with high nitrogen and chloride

excretion, followed by a period of normal output. Near the end there is again an increased excretion of water, salt, and nitrogen. The

TABLE IV.

Obstruction of Cardiac End of Stomach.
10 Per Cent Sodium Chloride Introduced Directly into Jejunum.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides		Nitrogen		
		mg	mg	mg.	cal per cent	cc.	per cent	gm	per cent	gm	
5–63 (wt. 17 kg.)	0	21.1	10 3	460	22.1						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	34.3	14.5	540	30.5	190	0.96	1.82	0.4	0.76	4 cc. 10 per cent NaCl per kg. in jejunum
	2	34.1	17.8	580	24.0	260	0 89	2.32	1.4	3.64	" "
	3	53.5	40.6	620	27.7	310	0.72	2.23	2.1	6.50	" "
	4					55	1.79	0.99	1.2	0.7	Dead
5–64 (wt. 17 kg.)	0	61.5	28 0	540	34.3						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	44 4	19 6	450	36.2	630	1 01	6.4	1.30	8 2	4 cc. 10 per cent NaCl per kg. in jejunum
	2	45 0	20 1	510	34 3	270	0 66	1 8	1 94	5 3	" "
	3	43 2	23 4	560	34.3	360	0 62	2.2	2.22	8 0	" "
	4					20	1.00	0 3	2.42	0 48	Dead
5–65 (wt. 18 kg.)	0	29.1	12 1	510	38.1						Operation. 4 cc. 10 per cent NaCl per kg. in jejunum
	1	50 4	25 2	460	29 6	1030	1 01	10.1	0.60	6 2	4 cc. 10 per cent NaCl per kg. in jejunum
	2	52 6	21 0	580	24 9	350	0 80	2 8	1 11	3 9	" "
	3	50 4	34 6	580	29 4	660	0 95	6 3	1 14	7 5	" "
	4	74 3	43 9	660	25 8	70	1 72	1 21	1 72	1 2	" "

animals survived 32, 36, and 45 days. At autopsy in each animal, the tape had cut through, thus reestablishing continuity. As a con-

trol on these experiments three animals were given 40 cc. per kilo of 2 per cent glucose. These show the changes characteristic of untreated animals with rapid death. The diuresis and salt and nitrogen excretion are even more marked than in untreated animals.

To determine the effect of sodium chloride given with a minimum of water on the toxemia of cardiac obstruction, three dogs were given

TABLE V.

Obstruction and Release of Cardiac End of Stomach.

No Treatment.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Urine					Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO₂ combining power	Amount	Chlorides		Nitrogen		
		mg.	*mg.*	*mg.*	*vol. per cent*	*cc.*	*per cent*	*gm.*	*per cent*	*gm.*	
5-44	0	32.1	19.6	510	36.2	65	0.72	0.51	1.23	0.86	Cardiac end obstructed
(wt.	1	49.2	25.2	500	33.4	50	0.10	0.05	1.20	0.60	" " "
15 kg.)	2	87.0	51.8	520	28.7	200	0.60	1.2	0.96	1.90	Obstruction released
	3	111.0	56.8	460							Died
5-46	0	25.2	11.2	500	35.3	60	0.37	0.22	1.16	0.70	Cardiac end obstructed
(wt.	1	35.3	27.3	490	32.4	555	0.41	2.30	0.30	1.74	
9 kg.)	2	91.5	45.6	470	28.7	225	0.60	1.35	0.78	1.80	Obstruction released
	3	134.0	65.2	430	12.4	25	0.17	0.05	0.62	0.20	Died
5-45	0	22.9	14.7	500	38.1	45	0.16	0.30	1.50	0.8	Cardiac end obstructed
(wt.	1	29.0	11.4	450	32.4	750	0.8	6.0	0.54	4.1	
16 kg.)	2	129.0	62.3	450	28.7	285	0.8	2.32	0.55	1.6	Obstruction released
	3	230.0	111.0	420	21.9	65	0.02	0.14	1.0	0.65	Died

4 cc. per kilo of 10 per cent sodium chloride directly into the jejunum, through a tube introduced when the cardia was obstructed (Table IV). These animals died in approximately the same time as untreated animals, but showed much less rise in the non-protein nitrogen than untreated animals.

Several experiments were made to determine the effect of sodium chloride after obstruction and subsequent release of the cardia. In

TABLE VI.

Obstruction and Later Release of Cardiac End of Stomach.
Treatment with 1 Per cent Sodium Chloride Solution Subcutaneously.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	mg.	mg.	vol. per cent	
5–41	0	34.1	15.4	520	45.7	Cardiac end of stomach obstructed
	1	34.1	15.4	490	34.3	
	2	40.0	21.0	510	32.4	Obstruction released. 40 cc. of 1 per cent NaCl per kg. injected
	3	75.0	45.5	560	28.7	
	4	34.9	8.4	510	29.6	40 cc. of 1 per cent NaCl per kg. injected
	5	28.0	16.1	560	28.7	40 " " 1 " " " " " "
	6	21.1	9.8	500	20.0	40 " " 1 " " " " " "
	7	26.1	9.8	600	21.9	40 " " 1 " " " " " "
	8	31.4	16.8	650	18.1	Died. Autopsy showed no cause for death
5–42	0	26.5	10.5	470	36.2	Cardiac end of stomach obstructed
	1	36.1	18.9	460	36.2	
	2	61.5	33.6	450	40.0	Obstruction released. 40 cc. 1 per cent NaCl per kg. injected
	3	32.3	14.0	510	36.2	
	4	22.8	7.0	470	41.9	40 cc. 1 per cent NaCl per kg. injected
	5	25.9	10.5	530	36.2	40 " 1 " " " " " "
	6	30.6	14.7	530	36.2	40 " 1 " " " " " "
	7	28.0	14.0	540	39.0	40 " 1 " " " " " "
	8	23.8	14.0	540	34.3	40 " 1 " " " " " "
	9	25.2	9.8	570	36.2	40 " 1 " " " " " "
	10	27.8	8.4	530	45.7	Normal
5–66	0	41.6	15.4	460	36.2	Cardiac end of stomach obstructed
	1	46.8	23.8	460	32.4	
	2	115.0	63.5	450	27.7	Obstruction released. 40 cc. 1 per cent NaCl per kg. injected
	3	38.1	19.2	490		
	4	25.7	11.2	540	50.2	40 cc. 1 per cent NaCl per kg. injected
	5	25.3	10.3	600	52.0	Normal

three animals, the tape was removed 48 hours after the cardia had been ligated. All died within 48 hours after the release. The non-protein nitrogen continued to rise after the ligature was removed (Table V).

In three animals 40 cc. of 1 per cent sodium chloride was injected subcutaneously each day after the removal of the obstruction (Table VI). In each instance the non-protein nitrogen, which had begun to rise before the ligature was removed, promptly fell to normal and

TABLE VII.

Obstruction and Later Release of Cardiac End of Stomach.
Treatment with 2 Per Cent Glucose Solution Subcutaneously.

Dog No.	Day after opera-tion	Blood (amount per 100 cc.)				Remarks
		Non-protein nitro-gen	Urea nitro-gen	Chlo-rides	CO₂ combin-ing power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
5–69	0	21.2	5.6	440	34.3	Cardiac end of stomach obstructed
	1	40.6	16.8	400	30.5	
	2	40.8	15.9	420	32.4	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	62.5	27.1	370	25.8	40 cc. 2 per cent glucose per kg. injected
	4	71.3	37.4	370	27.7	40 " 2 " " " " " "
	5	123.0	47.5	400		40 " 2 " " " " " "
	6	146.0	55.6	330		Died
5–72	0	27.5	10.7	430	36.2	Cardiac end of stomach obstructed
	1	29.4	14.0	500	34.3	
	2	97.3	48.1	460	29.6	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	152.0	74.3	460	38.1	40 cc. 2 per cent glucose per kg. injected Died
5–73	0	40.0	9.3	410	36.2	Cardiac end of stomach obstructed
	1	35.7	17.3	310	41.9	
	2	103.0	51.4	300	28.7	Obstruction released. 40 cc. 2 per cent glucose per kg. injected
	3	163.0	81.3	440	47.5	40 cc. 2 per cent glucose per kg. injected. Died

remained so. Two animals recovered. One died on the 8th day, autopsy revealing no cause for death.

As a control on this last group, three animals were similarly obstructed and released and given 2 per cent glucose solutions sub-cutaneously after the removal of the obstruction (Table VII). One

animal lived for 6 days, with a gradual increase in non-protein and urea nitrogen. This animal also had a low blood chloride. Two animals died in less than 96 hours with the changes characteristic of untreated animals.

DISCUSSION.

These experiments emphasize the very marked toxemia which develops after obstruction of the cardiac end of the stomach of the dog. The changes in the blood chloride are small as compared with those observed after pyloric obstruction. The administration of 1 per cent sodium chloride prevented entirely the toxemia and prolonged life indefinitely. This effect is not due to fluid alone as the same amount of 2 per cent glucose solution does not have any effect on the development of the toxemia. The chloride in the blood of animals so treated is lower than in untreated animals without an increase in chloride excretion.

Sodium chloride solution is of the greatest value in the treatment of the toxemia after it has developed if the obstruction is surgically released. Simple release and treatment with glucose solution do not change the course of the toxemia. The results obtained in this series are similar to those observed in the treatment of the toxemia resulting from obstruction of the upper gastrointestinal tract at other levels.

SUMMARY.

A study is reported of the effect of different methods of treatment on the toxemia of cardiac obstruction.

The average duration of life of untreated dogs is 3 days. Three dogs treated with 1 per cent salt solution subcutaneously lived 32, 36, and 45 days respectively without developing a toxemia. 2 per cent glucose similarly given, does not alter the course of the toxemia. Concentrated salt solution in small quantities given directly into the jejunum prevents the marked rise in non-protein nitrogen but does not materially prolong life.

Release of the obstruction does not change the course of the toxemia in untreated animals. The subcutaneous injection of 1 per cent sodium chloride solution after release of the obstruction causes a rapid return of the blood to normal and allows the animal to recover. A

similar amount of fluid given as 2 per cent glucose does not alter the course of the toxemia after release of the obstruction.

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THE BLOOD CHLORIDES IN PROTEOSE INTOXICATION.

By RUSSELL L. HADEN, M.D., and THOMAS G. ORR, M.D.

(*From the University of Kansas, School of Medicine, Kansas City, Kansas.*)

The characteristic chemical changes in the blood after upper gastro-intestinal tract obstruction are: a fall in chlorides, a rise in the CO_2 combining power, and an increase in the non-protein nitrogen and urea nitrogen (1). These changes have been interpreted by some as due solely to the increased loss of water and salts through vomiting and increased excretion into the intestinal tract with consequent dehydration and kidney retention. Others think there is a primary chemical intoxication which is at least one factor in the characteristic blood changes. Toxic substances may come from the intestinal mucosa, from the activity of bacteria within the lumen of the intestine, or possibly be formed in the process of protein disintegration throughout the body.

Whipple and his coworkers (2) have emphasized especially the rôle of proteoses in the intoxication. These may be isolated from the contents of the obstructed gut and arise, they think, from the intestinal mucosa. It has been shown that the symptoms characteristic of intestinal obstruction may be reproduced by the intravenous injection of the proteoses. Animals so injected show a rise in the non-protein nitrogen and urea nitrogen of the blood with an increased nitrogen excretion.

In certain other chemical intoxications such as in burns (3), x-ray intoxication (4), and lobar pneumonia (5), the chloride metabolism is similar to that observed in intestinal obstruction. These facts together with other observations in intestinal obstruction suggest that the chloride loss through vomiting and increased secretion into the intestinal tract may not entirely account for the changes in blood chloride. In seeking other possible causes for the loss in chlorides we have studied the blood chemistry of dogs with different types of proteose intoxication with special reference to the chlorides.

TABLE I.

Chemical Findings after Injection of Proteose from Intestinal Content of Dogs with Obstruction at Different Levels.

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides as NaCl	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
3-26	0	25.4	10.7	480	30.5	Intestinal content from dog with obstruction of jejunum
	2	31.6	17.5	460	18.3	Vomiting and diarrhea
	4	38.5	16.1	410	14.5	Died during night
3-50	0	25.0	12.6	480	32.4	Intestinal content from dog with obstruction of jejunum
	2	34.1	19.6	490	21.9	Vomiting and diarrhea
	4	42.2	23.8	460	17.2	Died during night
3-49	0	28.0	16.8	460	34.3	Intestinal content from dog with pyloric obstruction
	2	36.6	17.5	500	21.9	
	4	45.6	20.3	490	21.9	Vomiting and diarrhea
	24	38.9	18.2	480	26.6	Recovered
3-48	0	28.2	15.4	490	22.8	Intestinal content from dog with pyloric obstruction
	2	55.5	21.0	480	11.0	
	4	53.5	25.9	500	7.2	
	24	79.0	43.3	450	13.4	Died
3-32	0	25.0	12.6	470	39.3	Intestinal content from dog with cardiac obstruction
	2	37.0	22.4	470	5.0	Died
3-45	0	29.7	14.0	460	33.4	Intestinal content of dog with cardiac obstruction
	2	40.0	17.5	490	32.4	
	4	37.5	19.6	490	24.7	
	24	40.0	18.3	470	24.7	No symptoms

TABLE II.

Blood Chemical Findings after the Intravenous Injection of Witte's Peptone.

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
6-04	0	36.1	25.7	470	30.5	50 cc. 5 per cent peptone intravenously
(wt.	½	36.6	24.8	420	10.7	
12 kg.)	6	66.3	32.7	420	10.7	Died following night
6-05	0	33.3	16.8	540	32.4	50 cc. 5 per cent peptone intravenously
(wt.	¼	37.5	18.2	500		
15 kg.)	3	48.2	23.4	540	12.9	Died
6-18	0	30.0	14.5	520	30.5	50 cc. 5 per cent peptone intravenously
(wt.	½	43.6	22.4	480	9.2	
6.7 kg).	3	50.0	29.0	510	10.7	Died
6-02	0	38.5	17.3	500	40.9	50 cc. 5 per cent peptone intravenously
(wt.	¼	38.9	17.3	520	32.4	
8 kg.).	2	41.6	23.35	460		
	4	37.0	24.3	480	30.5	
	24	52.6	22.0	360	34.3	50 cc. 5 per cent peptone intravenously
	27	47.6	29.0	470	30.5	
	30	37.0	22.0	450	30.5	
	48	52.8	38.8	490	34.3	Recovered
6-73	0	30.0	7.0	490	37.2	50 cc. 5 per cent peptone intravenously
(wt.	20	20.3	4.7	470	32.4	50 " 5 " " " "
10.7 kg.).	44	26.3	4.7	470		50 " 5 " " " "
	68	24.2	8.4	440	36.2	50 " 5 " " " "
	92	25.0	9.3	380	33.4	50 " 5 " " " "
	116	28.7	6.5	480	38.1	50 " 5 " " " "
	140		45.8	440	39.0	50 " 5 " " " "
	146					Died

Method.

Proteose was prepared from the intestinal contents by the method described by Ellis (6) and dissolved in a small amount of distilled water for injection. All injections were made intravenously. The animals were bled from the jugular vein before injection and at intervals thereafter as indicated in the tables.

TABLE III.

Chemical Findings after Intravenous Injection of Proteose from Intestine of Dogs with Obstruction but without Toxemia (Treated with Sodium Chloride).

Dog No.	Hrs. after injection	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
3-23	0	28.5	12.6	410	25.6	
	2	33.3	19.6	410	12.6	
	4	36.1	17.5	400	18.0	No symptoms
3-27	0	31.2	14.0	470	36.2	
	2	40.0	18.9	460	10.1	Vomiting and diarrhea
	4	62.5	30.8	460	10.1	Died soon after
3-41	0	34.9	15.4	500	30.5	
	2	36.1	18.2	510	17.2	
	4	38.5	19.6	500	19.5	Vomiting
	24	30.3	13.1	500	27.5	Recovered
3-51	0	25.2	10.5	470	22.8	
	2	27.2	13.3	500	20.0	Vomiting and diarrhea
	4	25.4	11.9	500	14.3	
	24	26.1	11.9	490	20.0	Recovered

In the liver experiment liver was obtained with sterile precautions from one animal and placed free in the abdomen of another. All operations were done under ether anesthesia, with aseptic technique. The non-protein nitrogen was determined by the method of Folin and Wu (7), the CO_2 combining power by the method of Van Slyke and Cullen (8), the urea nitrogen by the Van Slyke and Cullen (9) modification of the Marshall method, and the chlorides on the tungstic acid filtrate in the manner described by Gettler (10).

EXPERIMENTAL OBSERVATIONS.

A large number of experiments were made. Typical results are shown in the tables. In Table I are recorded the blood chemical findings in animals injected with the proteose from other animals with obstruction at different levels. All show some increase in the urea nitrogen and non-protein nitrogen with a fall in the CO_2 combining power. Four of the six animals died within 24 hours. Most of them had vomiting and diarrhea. The chlorides show very little change.

TABLE IV.

Chemical Findings in Blood of Dog with Free Liver in Abdomen.

Dog No.	Day after operation	Blood (amount per 100 cc.)				Remarks
		Non-protein nitrogen	Urea nitrogen	Chlorides	CO_2 combining power	
		mg.	*mg.*	*mg.*	*vol. per cent*	
5-25	0	24.8	8.4	490	40.9	7.5 gm. of liver placed in abdomen
	1	29.1	9.1	490	40.0	
	2	32.6	12.2	480	34.3	
	3	27.0	8.4	490	32.4	
	4	27.3	7.0	490	32.4	
	5	34.5	10.5	570	30.5	Died, autopsy negative
5-33	0	41.6	18.9	500	36.2	12.5 gm. of liver placed in abdomen
	1	44.4	15.4	460	36.2	
	2	54.6	19.6	460	40.0	
	3	17.7	77.1	510	29.6	
	4	24.3	112.8	500	27.5	Died. No peritonitis. Small amount hemorrhagic fluid in abdomen

As a control on these experiments several animals were injected with Witte's peptone which consists largely of proteoses. These showed changes similar to those observed after injection of proteose from animals with experimental obstruction. The chlorides again showed no significant changes.

Animals dying of gastrointestinal tract toxemia usually show at autopsy a markedly distended gall bladder. Several dogs were injected with the proteose recovered from the bile of animals with

obstruction at different levels. One animal 24 hours after injection, showed a non-protein nitrogen of 124 mg. without significant changes in the chlorides. Two control animals and several others injected with material from obstructed animals showed very little change.

It has been shown that injections of sodium chloride solution will prolong the life of animals with high intestinal obstruction and prevent the development of a toxemia. Four dogs were injected with the proteose recovered from the obstructed intestine of dogs in which the development of a toxemia was prevented by the injection of salt solution. The results are shown in Table III. One animal showed a marked increase in non-protein nitrogen, two showed clinical evidence of intoxication with blood changes, and one showed no symptoms.

Davidson and Mason (11) found that a proteose intoxication could be easily produced in animals by placing liver free in the abdomen. The rise in non-protein nitrogen which takes place in such animals is due to the absorption of autolyzed protein. The blood chlorides were studied in two such animals. One showed very little change in the blood chemistry. One had a very great rise in urea nitrogen and non-protein nitrogen without significant change in CO_2 combining power or chlorides.

DISCUSSION.

The chemical changes in the blood produced by the injection of proteose were not so marked as those found by Whipple. This may have been due to the fact that a purified proteose was employed. In purifying the proteoses from the intestinal contents, either the proteoses became less toxic or some toxic substance is removed. In this series however, four of six animals injected died.

There is no definite change in the chlorides even with a marked rise in non-protein nitrogen. Likewise with a marked intoxication resulting from the injection of Witte's peptone, and in dogs with autolyzing liver within the abdomen, there is no significant change in the chlorides.

These findings would indicate that proteose intoxication is not concerned in the characteristic fall in blood chlorides observed after intestinal and pyloric obstruction.

SUMMARY.

Dogs injected with proteose recovered from the intestinal contents of animals with obstruction at different levels show no significant changes in the blood chlorides even with a fatal intoxication.

After the intravenous injection of lethal and sublethal doses of Witte's peptone there is little change in the chlorides.

Autolyzing liver in the abdominal cavity produces no change in the blood chlorides even with a very great increase in the urea and non-protein nitrogen.

Proteose intoxication is probably not a factor in the characteristic fall in chlorides seen after intestinal and pyloric obstruction.

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STUDIES ON A PARATYPHOID INFECTION IN GUINEA PIGS.

V. The Incidence of Carriers during the Endemic Stage.

By JOHN B. NELSON, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

The course of two Salmonella infections appearing spontaneously in a guinea pig population has been discussed in previous papers (1, 2). As noted earlier, the guinea pigs comprising the particular population are divided into two groups, breeders and weaned young or stock. Animals employed in experimental work are withdrawn from the latter group. From time to time *B. paratyphi* has been isolated from the spleen of stock guinea pigs injected with other bacteria. It seemed probable that the guinea pigs were carrying *B. paratyphi* in the spleen as a result of earlier exposure to infection.

A routine bacteriological examination of all guinea pigs that died had been carried out for several years. Positive cultures were rarely obtained from the spleen with unweaned guinea pigs in the absence of gross lesions. However, no definite study of carriers within the population at large had been attempted. Consequently a comprehensive examination of breeders and young guinea pigs, just prior to weaning, was undertaken. In addition a new breeding group composed of selected sows and boars was brought together and isolated from the main population. The selected group was kept under observation for evidence of reinfection.

Before considering the work on carriers a more detailed statement concerning the extent of the two infections may be pertinent. The specific percentage mortality for the population (including the selected breeders) during that year is given in Table I. The usual monthly fluctuations occurred. The rates for the second infection, introduced into the population a year earlier, were in general higher than those for the first form.

When the examination for carriers was begun the breeding stock was composed of some 209 animals. These were maintained in a separate animal unit, distributed in large metal cages containing 4 or 5 sows and 1 boar. The group was a heterogeneous one comprising animals from 1 to 5 years of age. A few of the number had passed through the active stage of the initial outbreak of paratyphoid in 1924. The majority of breeders, however, were younger animals added shortly before or at intervals after the appearance of the second infection during the summer of 1926. Generally the individuals in any one cage were of approximately the same age.

Cages containing a full quota of guinea pigs were selected and brought to the laboratory for examination. It may be said that pregnant sows are removed to

TABLE I.

Population, Total Deaths, Deaths from Paratyphoid, and Percentage Mortality from Paratyphoid from September, 1927, through June, 1928.

Month	Population	Total deaths	Deaths from Paratyphoid Type I	Mortality Type I	Deaths from Paratyphoid Type II	Mortality Type II
				per cent		*per cent*
September..................	513	11	1	0.19	6	1.16
October....................	561	17	3	0.53	11	1.96
November..................	668	23	1	0.14	5	0.74
December..................	573	27	2	0.34	3	0.52
January...................	661	60	3	0.45	13	1.96
February..................	631	39	2	0.31	6	0.95
March.....................	595	62	0	0.00	1	0.16
April.....................	497	73	1	0.20	1	0.20
May......................	493	43	2	0.40	1	0.20
June.....................	483	75	1	0.20	3	0.62

individual units just prior to parturition. In all, 105 animals were transferred, representing 20 cages. The entire work extended over a period of 5 months. Two successive fecal cultures were made from each guinea pig and the agglutinin content of the blood serum determined for the two types of *B. paratyphi.*

General Methods.

The following routine procedures were carried out.

The guinea pigs were first bled. In most instances sufficient blood was obtained from incising an ear vein after shaving and the application of xylol. 13 of the total number were refractory and heart puncture under ether was resorted to, with no fatalities. Serum from the coagulated samples was used in

testing. The antigens were fresh, standardized, saline suspensions of the two Salmonella types. The final dilution series in a total volume of 1 cc. ranged from 1:20 through 1:1280. The tubes were incubated at 37°C. for 3 hours and were read after overnight refrigeration. In Tables III and IV the results are expressed in terms of the limiting dilution, the highest dilution showing any macroscopic evidence of agglutination.

After bleeding, the guinea pigs were transferred to separate cages with clean bedding. The next morning a fecal sample was collected. The bedding was changed and on the following morning a second sample collected. The fresh feces, at least two pellets, were emulsified in 5 cc. of plain broth and incubated for 5 to 6 hours at 37°C. Two large loops were then transferred to a tube of malachite green-lead acetate broth.

Difficulty had been experienced with this medium in former work. Hydrogen sulfide in amounts detectable by lead acetate was produced only when fermented bouillon was employed. The peptone used was apparently deficient in the particular sulfur compound utilized for the production of hydrogen sulfide. With the fermented bouillon some available sulfur compound was evidently produced during growth of the fermenting bacteria. It may be noted that B. coli is added to the meat infusion and allowed to grow at incubator temperature. After steaming and filtering, peptone is added as usual. Tilley (3) recommended the addition of sodium thiosulfate to media employed for hydrogen sulfide production. This compound was substituted for fermented broth with excellent results. The medium as finally employed was beef infusion broth containing 1 per cent peptone, of an initial pH of 7.6, and tubed in 5 cc. amounts. Immediately before use there was added 0.05 cc. of a 5 per cent aqueous solution of sodium thiosulfate, 0.25 cc. of a 1 per cent solution of lead acetate, and 0.15 cc. of a 0.2 per cent aqueous malachite green solution. The addition of the lead acetate causes a bulky precipitate which settles rapidly. In this medium B. paratyphi and other members of the Salmonella group produce a uniformly turbid growth. The precipitate blackens during the first 24 hours and there is a distinct odor of hydrogen sulfide. Motility is retained but is less active than in plain bouillon. Other intestinal bacteria are generally restrained through 48 hours at 37°C. B. proteus, however, may develop with the production of hydrogen sulfide. Occasionally there is growth of unidentified bacteria with no change in color of the precipitate.

The tube of the above medium inoculated from the fecal broth culture was incubated through 48 hours at 37°C. In the absence of growth or in the presence of growth with no motility and no change in color of the precipitate the tube was discarded. In the presence of growth, motility, and a black precipitate a loopful was streaked on the surface of a Petri plate containing lead acetate-thiosulfate agar. A thin film of the same medium was layered over the inoculated surface. Under this semianaerobic condition B. paratyphi produces large dark brown colonies. In the presence of such colonies transfers were made from several to lactose and saccharose fermentation tubes. In the absence of acid and gas the

cultures were confirmed by agglutination with the two type antiserums. With each lot of fecal cultures a medium control inoculated from a young broth culture of *B. paratyphi* was included. The above procedure was followed in all the subsequent work.

The Incidence of Carriers among the Breeders as Determined by Cultural and Serological Examination.

Of the 105 guinea pigs examined 69 were added to the breeding stock after the appearance of the second infection. Only 4 were survivors of the initial outbreaks in 1924. An agglutination test with the two types of *B. paratyphi* as antigens was made on serum from each animal. Eight samples failed to agglutinate either type in the lowest dilution

TABLE II.

Number of Samples Showing Agglutination of One or Both Types of B. paratyphi at Each Dilution Interval.

Serum dilution	1:20			1:40			1:80			1:160			1:320			1:640			1:1280		
Type of bacillus	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both	I	II	Both
Number of samples	0	11	7	3	5	5	1	22	1	1	15	1	4	12	2	2	5	0	0	0	0

employed. The remaining samples agglutinated one or both of the antigens in dilutions ranging from 1:20 through 1:640. With 66 serums of the latter group the dilution was 1:80 or higher. A detailed classification of the samples as to limiting dilution for the major agglutinin is given in Table II. Only 5 of the 24 males included in the group examined showed an agglutinin titer higher than 1:80 as compared with 45 high reactors among the 81 females. Two serums which agglutinated one type through a dilution of 1:160 failed to agglutinate the other type in 1:20. The majority of serums, however, agglutinated both types generally with a difference of one or two dilutions between them. With 70 samples the major agglutinin (highest limiting dilution) was for Type II antigen as compared with 11 for Type I.

B. paratyphi was isolated from the feces of 3 guinea pigs of the group examined.

One was an old sow added to the breeding stock in the fall of 1924 shortly after the termination of the active stage of the initial infection. Type II *B. paratyphi* was cultivated from the feces on 2 successive days. The blood serum agglut'nated Type I antigen through 1:160, Type II antigen through 1:320. The animal was kept under observation for 3 weeks and then killed. The uterus contained 3 half-term fetuses. The surface of the spleen was scarred and pitted. Other organs were normal. Cultures from the spleen, placental tissue, and cecal feces were all negative.

The other positive cases were younger sows added to the breeding stock in December, 1926, and July, 1927, respectively, before and after the appearance of the second infection. The former was an active case. *B. paratyphi*, Type II, was isolated from the first fecal culture. The next morning the animal was weak with labored breathing and was killed. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:640. At autopsy a peritonitis was encountered. A heavy exudative membrane was reflected over the surface of the spleen and portions of the liver. The abdominal cavity contained a quantity of turbid fluid. Type II *B. paratyphi* was isolated from the spleen, liver, and cecal feces.

The second of the younger animals was a carrier. A Type II organism was cultivated from the feces on successive days. The blood serum agglutinated Type I antigen through 1:40, Type II through 1:80. After 2 weeks the animal was killed and autopsied. The uterus contained 2 nearly full-term fetuses. The spleen was atrophic and extensively roughened and pitted. Cultures from the spleen, placental tissue, cecal feces, and fetal spleens were all negative.

The Incidence of B. paratyphi among a Group of Selected Breeders.

All guinea pigs from the tested group which showed consecutively negative fecal cultures and with an agglutinin titer of 1:80 or lower were segregated as a nucleus for a new breeding stock. The carriers were killed, as noted. The guinea pigs which showed high agglutinin content were returned, in most instances, to their original unit and isolated in special cages. A few were killed and autopsied. The selected animals were isolated in a separate unit in groups numbering up to 5 sows and 1 boar. The two units were maintained independently.

During the 5 months occupied in testing the entire group additional fecal cultures were taken at intervals from the segregated individuals. At first, mass cultures were made from the breeding cages. During one examination which comprised eight cages a culture of Type I *B. paratyphi* was obtained from the feces of a single cage. The guinea pigs were immediately transferred to individual

pens and cultures made on 2 successive days. The positive culture was traced to a single sow from which the same type of organism was isolated. The animal was removed and later killed. At autopsy no gross lesions were encountered and cultures from the spleen, uterus, and cecum were all negative. The blood serum agglutinated Type I antigen through 1:160, Type II through 1:20. Fecal cultures from the other inmates of the cage were all negative.

For the third fecal examination the guinea pigs were isolated in individual cages and a single culture made. The group comprised 46 adult animals distributed in ten cages. In addition there were 22 younger breeders representing the second generation of the selected population. The entire lot of fecal cultures was negative.

The segregated group was maintained in the same manner as the main breeding stock. Pregnant sows were removed to individual cages shortly before parturition. Young guinea pigs were kept with their dam for 2 to 3 weeks and then weaned. The sow was returned to her original breeding cage. The young were separated as to sex and placed in large open pens. A few of the weaned young were used for experimental work. 9 of these were carefully examined subsequent to infection with other bacteria. Cultures from the entire spleen were negative throughout. The majority of them were allowed to mature for breeding purposes. Before admission to the breeding stock they were isolated in individual cages and fecal cultures made.

The selected population was kept under observation and all animals that died were autopsied and cultured. Through June, 1928, a total of 111 guinea pigs was examined. The majority of them were still-born or unweaned young. *B. paratyphi* was isolated from the spleen on four occasions.

The first culture was obtained in February from a female stock guinea pig. At autopsy a large amount of turbid fluid was found in the pleural and peritoneal cavities. The spleen was enlarged and coated with a heavy, tenacious exudate which was likewise reflected over portions of the liver. The cervical and peritoneal lymph nodes were enlarged and congested. Type I *B. paratyphi* was isolated from the spleen, gall bladder, pleural fluid, and cecum.

The second culture was isolated from an unweaned male guinea pig whose dam had died on the previous day. At autopsy the lymphoid tissue of the cecum was congested and a culture of Type II *B. paratyphi* was secured from the spleen. The dam showed no evidence of infection. The spleen failed to show *B. paratyphi*.

The third and the fourth cultures were isolated in June, in both cases from sows. At autopsy, the first animal showed an enlarged and congested spleen. Both spleen and liver were coated with a tenacious exudate. The gall bladder contained purulent fluid. There was an inflammation of the small intestine. The abdominal cavity contained a mucoid fluid. Type II *B. paratyphi* was isolated from the spleen. The second sow was carrying 4 nearly full-term fetuses. No gross manifestations of infection were encountered. Type I *B. paratyphi* was cultivated from the spleen.

The Incidence of B. paratyphi among Unweaned Young of the Main Population.

A less extensive study was made of the incidence of carriers among the unweaned young of the main population.

Monthly examination of 6 guinea pigs from as many litters, chosen at random, was carried out during the period between September, 1927, and February, 1928. The animals varied in age from 2 to 3 weeks. They were brought to the laboratory, bled from the heart under ether, and killed. Postmortem examination was made and cultures taken from the spleen, gall bladder, small intestine at a Peyer's patch, and cecal feces.

During October, 2 fecal carriers and 1 active case of paratyphoid were encountered. All 3 were females, 3 weeks old. Type II *B. paratyphi* was isolated from the cecum of the 2 carriers. The abdominal organs showed no gross changes. Cultures from the spleen, gall bladder, and small intestine were negative in each instance. The active case showed typical gross changes in the abdominal viscera. The spleen was enlarged, congested, and showed a single nodular focus. The liver showed a few small foci. There were numerous exudative plaques on the surface of the cecum with foci in the lymphoid tissue at the head. Type II *B. paratyphi* was obtained from the spleen, small intestine, and cecal feces. During the same month 3 active cases occurred among the young of a second sow from the same breeding cage. In February and March fatal cases were noted among the young of 2 sows originally caged with the dam of the first carrier, mentioned above. All of the unweaned guinea pigs examined during the remaining months were normal with negative cultures throughout.

Agglutination tests were made on blood samples from all the unweaned guinea pigs. The procedure previously outlined was followed except that the dilution ranged from 1:10 through 1:640. The results expressed in terms of the limiting dilution showing agglutination are given in Table III. Seven samples failed to agglutinate either type of antigen in the lowest dilution. Four samples agglutinated both types through the same limiting dilutions. The remainder agglutinated both types of antigen but the limits of reaction varied by one or more dilutions. With eighteen samples of this number the major reaction was against Type II, while with seven the major reaction was against Type I. Classified according to sex it may be noted that serums from 2 of the 12 males and 5 of the 24 females were negative. Serum from the first of the fecal carriers agglutinated Type I through 1:20, Type II through 1:80; that from the second agglutinated Type I through 1:20, Type II through 1:40. There was no agglutination against either type with serum from the active case.

Agglutination tests were also made on serum from the dams of 3 unweaned guinea pigs and from the remaining young of their litters. The findings, which suggest maternal transmission of antibodies to the suckling young, are given in

pens and cultures made on 2 successive days.   The positive culture was traced to a single sow from which the same type of organism was isolated.   The animal was removed and later killed.   At autopsy no gross lesions were encountered and cultures from the spleen, uterus, and cecum were all negative.   The blood serum agglutinated Type I antigen through 1:160, Type II through 1:20.   Fecal cultures from the other inmates of the cage were all negative.

For the third fecal examination the guinea pigs were isolated in individual cages and a single culture made.   The group comprised 46 adult animals distributed in ten cages.   In addition there were 22 younger breeders representing the second generation of the selected population.   The entire lot of fecal cultures was negative.

The segregated group was maintained in the same manner as the main breeding stock.   Pregnant sows were removed to individual cages shortly before parturition.   Young guinea pigs were kept with their dam for 2 to 3 weeks and then weaned.   The sow was returned to her original breeding cage.   The young were separated as to sex and placed in large open pens.   A few of the weaned young were used for experimental work.   9 of these were carefully examined subsequent to infection with other bacteria.   Cultures from the entire spleen were negative throughout.   The majority of them were allowed to mature for breeding purposes.   Before admission to the breeding stock they were isolated in individual cages and fecal cultures made.

The selected population was kept under observation and all animals that died were autopsied and cultured.   Through June, 1928, a total of 111 guinea pigs was examined.   The majority of them were still-born or unweaned young.   *B. paratyphi* was isolated from the spleen on four occasions.

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Agglutination tests were also made on serum from the dams of 3 unweaned guinea pigs and from the remaining young of their litters. The findings, which suggest maternal transmission of antibodies to the suckling young, are given in

TABLE III.

Limiting Agglutination with Serum from Unweaned Guinea Pigs.

Month	Type of bacillus	Limiting dilution of serums					
Guinea pig No................		1	2	3	4	5	6
September	I	40	20	40	80	20	10
	II	80	40	40	40	80	20
October	I	20	20	20	20	—	—
	II	20	80	40	80	—	—
November	I	20	40	20	40	20	20
	II	80	20	80	10	80	160
December	I	80	40	80	10	—	—
	II	20	80	40	20	—	10
January	I	—	—	10	—	10	10
	II	—	—	20	—	20	20
February	I	20	20	—	10	80	20
	II	80	—	—	10	20	20

TABLE IV.

Agglutinin Titer of Serum from Sows and Their Unweaned Young.

Class of guinea pig	Limiting dilution	
	Type I	Type II
1st young...	10	10
Sow...	80	20
2nd young..	20	10
3rd "	20	10
1st "	80	20
Sow...	160	20
2nd young..	40	10
3rd "	40	10
1st "	20	80
Sow...	40	320
2nd young..	20	80
3rd "	20	80

Table IV. It may be said that all the animals were later killed with negative pathological and bacteriological findings throughout. There was an interval of 4 days between the examination of the first young and the other guinea pigs of the group.

DISCUSSION.

The examination of breeders described in the foregoing pages was begun in October, 1927. 13 months earlier the second type of Salmonella infection had appeared spontaneously in the population. There had been a slow but general spread of the second bacterium throughout the breeding group. Specific deaths occurred among the sows or the unweaned young of sows from 32 of the 48 cages. The Type I infection had long since become endemic, with the mortality rate in general on a low level.

The serological findings appear to reflect the distribution of the two Salmonella types. 92 per cent of the 105 serums tested showed well defined agglutination against one or both types of *B. paratyphi* in dilutions ranging from 1:20 through 1:640. With 86 per cent of the reactors the higher agglutination was against the second, more recent type of organism. With 13 per cent the higher agglutination was against the initial form. In each case the majority of serums agglutinated both types although one in lower dilution. The physical form of the sediment was not sufficiently defined to permit a distinction between common agglutinins for the one type and specific agglutinins for the other.

It was assumed that the high agglutinin content of individual serums was referable to a multiplication of one or both strains in the system of the guinea pig. Postmortem cultures from a small series of high reacting guinea pigs failed, however, to demonstrate the specific organism in the spleen, liver, or section of the small intestine. These observations suggest that a high agglutinin titer may be maintained in the absence of a persistent localization of *B. paratyphi* in the more usual sites. The bacteriological examination, however, was not exhaustive. The entire spleen was cultured but examination of the liver and small intestine was necessarily limited. Moreover, the possible localization of the specific bacterium in regional lymph nodes is not ruled out. Temporary colonization of *B. paratyphi* in one or another of these sites might well impart the stimulus leading to antibody formation.

The serological findings with the unweaned guinea pigs roughly parallel those with the breeders. The total number of reactors while high was less than with the mature animals, 80 per cent as compared with 92 per cent. The young guinea pigs also showed a greater number of high reactors against the second type of *B. paratyphi* than against the first. The difference was not as marked, however, as with the older animals. The agglutinin titer in general was on a lower level than that of the breeders. The highest limiting dilution recorded was 1:160, with the majority of serums well below that figure. A number of serums from the breeders agglutinated through 1:640 with a fair proportion showing reaction between 1:80 and 1:320. There was a suggestion that the agglutinin content of the young was referable, in part, to placental transmission or to lactation. Sublethal infection at birth with immunization was not excluded as a possible cause of the presence of agglutinins in the group at large.

By way of comparison it may be of interest to note the serological findings reported by Amoss (4) on the survivors from an artificially induced paratyphoid epidemic in mice. A population of 300 mice comprising susceptible individuals in contact with a previously infected group was set up. During the epidemic wave which followed, 69 per cent of the population died. He tested the serum from 56 of the survivors for agglutinin against the causal organism, Mouse Typhoid II. The serum from 37 or 66 per cent partially agglutinated the organism in dilutions ranging from 1:20 to 1:160. The serum from 27 showed no agglutination in 1:20.

The cultural tests in the present work indicated a low incidence of fecal carriage among the breeders. *B. paratyphi* was isolated from the feces of only 3 sows, 1 of them an active case. A group of 50 guinea pigs selected from the total number examined was again cultured on three occasions with the detection of a single additional carrier. While the results cannot be regarded as strictly quantitative it is believed that the figure cited is a fair index of the extent of specific fecal excretion in the breeding group as a whole. Some of the animals examined were selected from cages in which fatal cases of paratyphoid had previously occurred. Conditions were favorable for the ingestion of the specific bacteria with food soiled by fecal excretions. In the face of such conditions the small number of fecal carriers actually detected

seems to bear out the suggestion made in a previous paper that specific fecal excretion with adult guinea pigs is of relatively short duration (5).

Examination of the unweaned young showed that fecal carriage of *B. paratyphi* may occur with nursing guinea pigs in the absence of generalized infection. *B. paratyphi* was isolated from the feces of 2 guinea pigs which showed no manifestations of disease. The group tested was too small to indicate the actual extent of such carriage. The young guinea pigs after weaning are transferred to the general stock for laboratory use. The presence of fecal carriers among them may account for the subsequent occurrence of sporadic cases or carriers. It is known that *B. paratyphi* may localize in the spleen or liver with no indication of multiplication or injury. It is possible that the introduction of other bacteria into such guinea pigs may be followed by multiplication of the paratyphoid types with active disease.

Both types of *B. paratyphi* appeared among the guinea pigs of the segregated group. The selected population which included breeders and later unweaned young and stock was maintained in a separate unit. There was no communication with the main supply of guinea pigs. Chance contact with field animals was practically impossible. Introduction of the specific bacteria with the food was not entirely ruled out. It seemed more probable, however, that the initial selection was not sufficiently rigid and that a number of spleen or fecal carriers were admitted to the new breeding stock. To date (June, 1928) there has been no evidence of extensive dissemination of the bacteria throughout the population.

SUMMARY.

A group of 105 breeders and 36 unweaned guinea pigs was tested to determine the extent of specific fecal excretion and the proportion of serum reactors in a population naturally infected with two types of *B. paratyphi*. The second, more recent type of organism was isolated from the feces of 3 breeders and 3 young. No carriers of the first type were detected. 86 per cent of the breeders and 72 per cent of the unweaned guinea pigs agglutinated the second type of *B. paratyphi*

in dilutions ranging from 1:10 through 1:640.   13 per cent of the breeders and 28 per cent of the unweaned young agglutinated the initial type.   There was a cross or double agglutination in most instances.   The serological findings roughly reflected the distribution of the two types as indicated by the mortality rate of the population at large.

50 breeders selected on the basis of agglutination and fecal examination and therefore supposedly free from infection were segregated and kept under close observation.   Both types of *B. paratyphi* subsequently appeared in the group.

During this time carriers were discovered by others in the Department among the stock guinea pigs used for other experiments in that cultures of the entire spleen were positive in perhaps 5 to 10 per cent of the guinea pigs so used.

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STUDIES IN THE BIOLOGY OF METALS.

VII. The Influence of Lead on the Development of the Chick Embryo.

By FREDERICK S. HAMMETT, Ph.D., and VILMA L. WALLACE, M.D.

(From the Research Institute of The Lankenau Hospital, Philadelphia.)

INTRODUCTION.

A knowledge of the fundamental biological reactions to metals is a prerequisite to their intelligent use in the fight against disease. It has long been known that lead retards growth, but the studies so far made have dealt merely with the gross response (1–5). They are thus inadequate save as confirmations of a predictable reaction. They tell nothing of the differential response, or whether it is growth by increase in cell size or growth by increase in cell number which is affected. This distinction is of importance if the use of lead or any other metal as an inhibitant of pathological growth is to have a rational basis.

As a first step in the analysis of this phase of metal activity a series of studies was made of the influence of lead on cell growth and mitosis in root tips. The results are reported in preceding papers. Briefly, it has been established by this work that lead retards growth because it inhibits mitosis or cell proliferation, not because it inhibits cell growth in size (6). It does this because certain chemical properties of the nucleus, particularly the nucleus in mitosis, make possible the concentration of lead therein (7, 8), thus precipitating out of action substances essential for cell division (9).

While there is every reason to suppose from both chemical and morphological evidence, that the phenomenon of mitosis is fundamentally the same in plants and animals, and hence that the basis of growth inhibition by lead would be the same in each, yet supposition is not proof. For this reason a repetition of the plant experiments

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was begun on the animal. The chick embryo was chosen because material is at all times available, because controlled cultivation is possible, and because its developmental stages are well known and accurately determinable by somite count.

Procedure.

A series of embryos was subjected to lead during growth as follows: Clean, unincubated eggs were lightly brushed with 80 per cent alcohol. They were allowed to stand under sterile conditions for 10 or 15 minutes until the rotation of the yolk had brought the blastoderm to the top. The egg was then lightly and quickly flamed with an alcohol lamp and placed on an appropriately designed sterile holder. The top surface was dabbed with alcohol and a window cut in the shell with a safety-razor blade. The blastoderm now being in view, 0.4 to 0.5 cc. of yolk was removed and replaced with the same volume of $Pb(NO_3)_2$ solution containing 0.05 per cent Pb ion. This was done with a micro syringe. Care was taken to avoid injury through stretching of the yolk sac during the puncture, or through making the injection too rapidly or too near the blastoderm. The lead was injected under the blastoderm through the same hole that was used for the withdrawal of the yolk. The membrane and shell were then replaced and the edges sealed with paraffin. Precautions for the maintenance of sterility were rigorously taken. The egg was then placed in the incubator with the window *not* on top. By this detail the embryo is brought to the top of the egg out of the zone of tension caused by the cut in the external membrane. It thus escapes mechanical distortion which much experience has shown to be the cause of abnormal development and high mortality.

After incubation for 18 to 72 hours the eggs were taken from the incubator. The living embryos were gently cut from the yolk sac, washed free from yolk in warm saline with brush and pipette, dehydrated in increasing concentrations of alcohol, cleared in xylol, stained with neutral red and Janus green, and mounted in balsam. In this way a series of leaded embryos from the head fold to the 30 somite stage was obtained.

For comparison three sets of controls were prepared. The first consisted of normal embryos, the second of embryos from eggs in which the window alone had been cut, and the third of embryos subjected to 0.8 per cent NaCl instead of lead. The reaction to lead was determined by comparing the tests with the controls of equal somite number. This, rather than the time of incubation, is the accepted index of degree of development in the early stages.

Fresh, clean, fertile eggs from the White Leghorn and Barred Plymouth Rock breeding stock of the Pratt Experiment Farm were used.
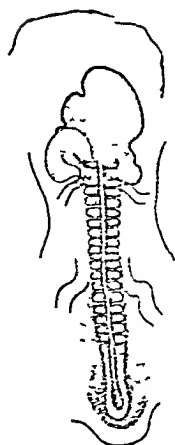
FIG. 1 a. Leaded embryo (1-4′) of 20 somites.
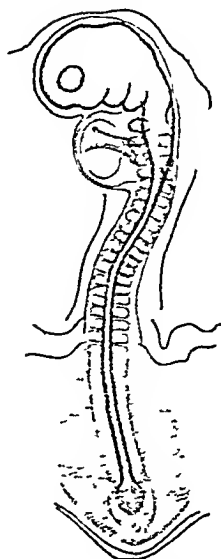
FIG. 1 b. Normal embryo (N-8) of 20 somites.
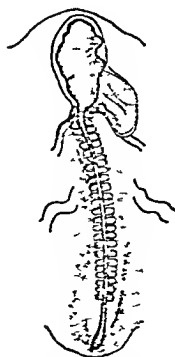
FIG. 2 a. Leaded embryo (L-9) of 26 somites.
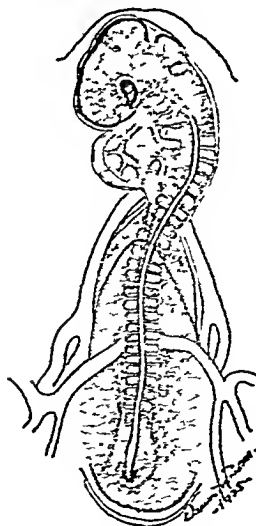
FIG. 2 b. Normal embryo (N-12) of 26 somites.

Drawings of normal chick embryos and of embryos subjected to lead during development.

The most striking response to the presence of lead is the retardation of the differential development of the head end of the embryo. This is exhibited in the youngest head fold stage as well as in the one of 30 somites or more. The phenomenon is shown in Figs. 1 *a*, 1 *b*, 2 *a*, and 2 *b* where leaded embryos of 20 and 26 somites are compared with their normal controls. The absence of progressive differentiation is marked in these two characteristic specimens. In less affected embryos, even though growth has proceeded, the retardation of development of the optic anlagen is clearly evident.

This selective type of inhibition does not follow the disturbance caused by cutting the window, nor when there is added to this the removal of 0.5 cc. of yolk and its replacement with saline solution. In the latter case, indeed, the impression is given of a tendency to an overdevelopment of the head region. This was not confirmed by detailed study since our interest was primarily in the lead reaction.

The somites are also the seat of a differential retardation. When the width and length of these active centers of cell proliferation are measured in embryos which develop in contact with lead, it is found that they are not only absolutely, but also relatively smaller than the same placed structures in the controls of equal somite count. This is determined by the quotient of embryo dimension into somite dimension. The data over a wide range of development are given in Table I.

The consistency of the difference at the several stages establishes the reaction as definite. Its significance is determined by the fact that it appears neither in the window controls nor in the saline controls. In other words, lead produces a specific differential retardation of somite growth.

A third result is that the embryos which grow in contact with lead are smaller than their controls of equal somite number. This retardation of gross growth is of course what was to have been expected. It is essentially specific in that it does not occur when the eggs are only opened, or opened and injected with saline.

A distinction needs to be made here between embryo size as based on somite count, and size as based on duration of incubation. The

TABLE I.

Somite and Embryo Dimensions in Lead-Treated Chicks and Their Controls.

Embryo No.	Somites measured	Somite		Embryo		S/E	
		Width 0.01 mm.	Length 0.01 mm.	Width 0.01 mm.	Length 0 01 mm.	Width	Length
						per cent	per cent
				15 somites			
Pb-L3	13	8.1	8.2	109.6	260.3	7.4	3.2
Cont.-N7c	13	13.2	10.3	123.3	287.7	10.7	3.6
				21 somites			
Pb-L4	14						
Pb-L4′	15 av.	8.9	8.6	123.3	311.0	7.2	2.8
Pb-L5	12						
Cont.-N8	14						
Cont.-N9	10 av.	12.6	12.1	141.1	397.3	8.8	3.0
Cont.-N10	10						
Cont.-S8	14	13.4	11.4	150.7	383.6	8.9	3.0
Cont.-W14′	10						
Cont.-W15	13 av.	15.9	11.9	178.1	411.0	8.9	2.9
				23 somites			
Pb-L7	10						
Pb-L7′	14 av.	11.4	10.7	143.9	404.2	7.9	2.6
Cont.-N11	12						
Cont.-N11′	14 av.	15.3	15.2	171.3	534.3	9.0	2.8
Cont.-S9	13						
Cont.-S9′	15 av.	12.1	12.3	130.2	417.9	9.3	3.0
Cont.-W16′	11						
Cont.-W17	12 av.	15.1	13.7	164.4	438.4	9.2	3.1
				26 somites			
Pb-L8	10						
Pb-L9	20 av.	12.9	10.4	134.3	387.7	9.6	2.7
Pb-L9′	11						
Cont.-N11a″	14						
Cont.-N12	13 av.	18.5	16.4	160.3	524.7	11.5	3.2
Cont.-N13	10						
Cont.-S10	11	17.1	17.1	157.6	534.3	10.9	3.2

Pb—Lead-subjected embryos.
Cont.-N—normal controls.
Cont.-S—saline controls.
Cont.-W—window controls.

disturbance due to operation does slow down growth so that it takes a longer time for the embryo to reach the same somite number as the undisturbed control. But notwithstanding this delay, when the embryo does reach a given somite stage of development it is essentially the same size as its normal control of somewhat younger age. This is shown in the table. On the other hand, the leaded embryo is not only delayed in reaching normal somite count, but also when this is reached after the longer interval of incubation, it has failed to attain the dimensions normal for the somite stage of development. Hence it is obvious that a specific type of gross growth retardation is produced by lead.

SUMMARY AND CONCLUSIONS.

Our study of the effect of the lead ion on the development of the chick embryo has brought out the following facts:

1. Gross growth is retarded.

2. Somite growth is retarded to a degree greater than that exhibited by body length and width.

3. The head and optic anlagen are regions of particular sensitivity. Their differential development is markedly inhibited.

From the purely biological point of view these results are in line with the findings of Child (10) and his school as to the sensitivity of the head end of rapidly growing organisms to harmful influences, and with those of Stockard (11) as to the peculiar sensitivity of the optic anlagen.

It is almost too well known to need repetition that the head region and the somites of embryos are specific areas of intense growth by increase in cell number. Therefore, turning from the general to the particular, the differential retardation of these regions which is caused by lead, is evidence justifying the conclusion that it is areas of rapid growth by cell proliferation which are selectively inhibited by this metallic ion.

Credit for the drawings is gratefully given to Eleanor Paxson, artist to the Research Institute.

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STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

II. The Action of Light on Erythrocytes in Vitro.

By W. R. EARLE.*

(*From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville.*)

In the course of a series of studies on white blood cells exposed to the action of light, it was found that they underwent a rapid and extreme degeneration (Earle (4)). It has also been found that red blood cells, when exposed to light, undergo degenerative changes, the cell swelling, and finally undergoing hemolysis.

The fact that red blood cells of the beef may be hemolyzed through the action of light from the carbon arc, was shown by the work of Schmidt-Nielsen (10) in 1906. A similar hemolysis was also demonstrated in the same year by Busck (1), who used the blood of the rabbit and irradiated it with light from a carbon arc. Busck also demonstrated that the rays capable of causing this hemolysis were filtered out by glass, and therefore lay in the ultra-violet region of the spectrum. Busck considered that some of his experiments suggested that oxygen played a definite rôle in this hemolysis.

Dreyer and Hanssen (3) illuminated human erythrocytes by means of light from a Bangs lamp, having cooled silver electrodes, and found that, once hemolysis had begun, the number of cells hemolyzed each minute coincided with the number which would be hemolyzed if the reaction were a monomolecular one. In this work the red cells were suspended in saline, the cells being placed on a hemocytometer slide and covered with a quartz cover-slip, through which the light was directed.

Schmidt-Nielsen (11) irradiated erythrocytes of the beef by means of light from

a carbon arc, and found that the light sensitized the cells to hypotonic solutions, instead of producing a true primary hemolytic action. He found that hemolysis of unirradiated cells did not occur until the salt content of the solution was reduced to about 0.6 per cent NaCl, whereas, after irradiation, continued hemolysis occurred when the salt concentration was lowered to 0.9 per cent NaCl. When the cells were placed in solutions of high salt content, however, this hemolysis did not occur.

Hasselbalch (5) had his attention called to the action of light on blood through the lessened oxygen capacity of blood which had been exposed to light. He showed that this diminution in oxygen capacity was the result of a transformation of hemoglobin into methemoglobin. Hasselbalch also found that while the light wave-lengths, most active in causing hemolysis, were those shorter than $310\mu\mu$, there was some slight action by longer wave-lengths, transmitted by glass. He also found that this hemolysis, as a result of irradiation, occurred even in a high vacuum, and concluded that the presence of air was not essential for its occurrence.

Koeppe (6), using human blood, verified this action of the ultra-violet region of the spectrum in causing a transformation of hemoglobin to methemoglobin, and in causing an hemolysis of the red cells, but believed that this hemolytic action was limited to wave-lengths shorter than about $270\mu\mu$. From his results he considered that it was the action of light on the shells of the red cells which caused their hemolysis. Koeppe also emphasized the observation that a relatively short irradiation of the red cells caused very slight immediate hemolysis, but that after the elapse of some time without further irradiation, this hemolysis was greatly increased. Koeppe considered that there was an oxidation of the lipoid material of the shell of the cell, during the actual process of irradiation, this oxidation occurring in the presence of oxygen, and resulting in the formation of acids which, in turn, caused continued hemolysis of the red cells after the irradiation had been discontinued. Koeppe was able to show the existence of some substance in the suspending fluid, in which red cells had been irradiated, which was able to hemolyze unirradiated red cells.

Koeppe (7) also demonstrated that samples of children's blood, drawn immediately after a 30 minute irradiation of the children with a quartz mercury vapor lamp, were markedly more susceptible to hemolysis by light than were samples drawn before the children had been irradiated.

Reed (9), irradiated the blood of the dog with the tungsten arc, the light reaching the blood by passing through the wall of a quartz tube inserted in the course of the carotid artery. He found that only minor changes occurred in the hemoglobin concentration of the blood, and in the erythrocyte count.

Clark (2) irradiated the ears of rabbits with light from the iron arc and also noted only minor changes in the hemoglobin and in the erythrocyte count.

Miles and Laurens (8) irradiated dogs 1 hour a day for 8 days, using the carbon arc as a light source, and found an initial increase in the red cells. This increase was later followed by a decrease.

Materials and Methods.

The observations were made on hanging drops of blood obtained from rabbits. A drop of blood was drawn by pricking an ear vein. A cover-slip was touched to this drop, removed, and given a slight jerk so that the blood adhering to the cover-slip was spread out into a streak. The cover-slip was then inverted over a hollow ground slide, and its edges sealed with "Salvoline." The culture was placed at once in a dark water-jacketed incubator kept at 39°. This entire procedure was carried out in dim light, and as none of the preparations was kept longer than about 3 hours, no special effort at asepsis was made. These preparations were made in groups of three or four, one preparation being irradiated and simultaneously observed, the other two or three in the set being kept in the dark as controls.

In the irradiation of these cultures, the same light source and optical system used in the work previously reported (reference 4: spectrum 4; Fig. 4) were employed. Irradiation was begun within 10 minutes after the preparation of the culture. The culture was kept under constant observation during the whole time of irradiation.

EXPERIMENTAL.

A representative culture showing the degeneration of the red cells under the action of light is presented below. Where reference to the degeneration of the leucocytes is made, discussion has been limited to the polymorphonuclear neutrophil, which is used as a typical example.

Experiment 1.

Culture 1572.—Irradiation was begun. The red cells were normal at this time: the leucocytes were moving around slowly. *30 minutes later:* The red cells were markedly swollen and appeared practically biplanar in profile. *40 minutes:* The red cells were even more swollen, the neutrophils were moving slowly and irregularly. *55 minutes:* Within the last 5 minutes a complete hemolysis of all red cells within the irradiated area had occurred. When examined in gross at this time the slide showed an area of complete hemolysis.

Culture 1573.—Control culture, kept in the dark. *60 minutes after inoculation:* The red cells were not swollen, nor were there any gross signs of hemolysis. The neutrophils were apparently quite normal and were moving around fairly rapidly.

The process of degeneration of the red cells, under the action of light, as observed in these cultures, took place as follows: In from 15 to 30 minutes after irradiation had begun, the red cells in the irradiated area of the culture showed marked swelling. At first they

looked merely a trifle thicker than normal, especially around their periphery. The swelling continued however, and the erythrocytes gradually lost their biconcave profile and became first almost bi-planar in profile, then biconvex. In a short time they had become perfectly spherical, and also more turgid, as indicated by an increased resistance of the cells to deformation. However, in spite of the swell-ing and increased turgor, there was only a slight increase in the greatest diameter of the cells. This process of swelling required from 30–90 minutes.

During the early part of this process there was no appreciable liberation of hemoglobin. During the latter part the results were inconstant, but in the majority of instances there was a gradual liberation of hemoglobin from the cells. This appeared to take place from all of the cells simultaneously. Often however, there was no trace of such an hemolysis during the swelling process. In such in-stances, a short time after the cells had become spherical, there was a sudden liberation of large concentrations of hemoglobin from the cells into the surrounding solution. This liberation appeared to take place simultaneously from all of the cells, and it often took place so suddenly that while at the beginning of the liberation process, the cells were normal in color, 3 minutes later not a single pigmented red cell could be distinguished in the whole microscopic field. All had been reduced to achromatic shadowy shells, while the culture medium had a deep orange tinge from the liberated hemoglobin. This com-plete process of liberation required about 90–110 minutes.

In the cells of a few cultures another modification of the process of liberation of the hemoglobin was noted. In these, the release occurred very gradually and never went to completion, as the cells remained of a distinct red color. Upon continued irradiation, instead of further liberation of pigment, the red cells took on the appearance of having been coagulated. They became less translucent than the normal cells and their outlines became increasingly hazy.

Hanging drop preparations similar to those above described, were made in the course of some other experiments. In these, however, the blood was diluted with 5–10 volumes of unbuffered or buffered Locke's solution. Upon irradiation of these preparations a strikingly different picture of degeneration was noted. As before, the cells be-

came swollen, and finally spherical, but following this, instead of a partial hemolysis of the cells, accompanied by a coagulation, or a sudden, almost explosive hemolysis of all of the cells exposed to light, there occurred a complete hemolysis of the cells, one or two at a time. This process was continued until finally, in from 15 minutes to 4 hours after the initiation of hemolysis, every cell in the field appeared as only a colorless shadow or "ghost" cell. Typical protocols from this series may be seen in Experiment 3 (page 672).

In the actual process of liberation of the hemoglobin, in both the diluted and the concentrated blood, no change could be observed in the walls of the red cells, and there was certainly no sign of gross rupture. Apparently the liberation of pigment was due to some submicroscopic change, either in the cell contents, the cell wall, or both, this change being such as to allow the pigment to leak out through the cell wall.

The next point considered was whether the range of wave-lengths of the light rays causing this hemolysis coincided with the range causing the degeneration of the leucocytes, as described in the previous paper. To determine this, hanging drops of blood were employed, as in the preceding experiment, and the same light source was used as formerly. Wratten filters Nos. 29, 45 and 58, and a 5 per cent total transmission neutral filter were consecutively substituted for the Corning daylight glass in the optical system. Wave-lengths of light transmitted by each of the filters employed were very active in causing hemolysis of the red cells. Further, the rate of degeneration of the cultures when irradiated under any one of the three colored filters, was practically the same. Nor did the rate vary from the rate shown when the culture was irradiated without a filter or with a 5 per cent total transmission neutral filter.

In all of these preparations serum had been present in some quantity, and the question next came up as to whether its presence was a factor conditioning the appearance of this degeneration of the red cells under the action of light. In order to obtain data on this point, 1 cc. of blood was drawn from an unanesthetized rabbit, by cardiac puncture. This blood was immediately mixed with 15 cc. of unbuffered Locke solution and then centrifuged at low speed in order to separate the cells. In this way the red cells were washed eight

times.   The cells were then suspended in 10 cc. of unbuffered Locke solution and hanging drop preparations were made as before. Upon irradiation of these hanging drops, complete hemolysis of the cells occurred as usual, except that it took place with greater rapidity than in the hanging drops of whole blood, and in a manner similar to that described for the irradiated cells of whole blood diluted with Locke solution.   From this it would appear that serum is not essential for the degeneration of the red cells under the influence of light.   The protocol of a representative culture of this series is presented below.

Experiment 3.

Culture 1625.—Washed red cells, suspended in Locke solution.   Upon first examination the erythrocytes appeared normal.   Irradiation was begun.   *9 minutes later:* Every cell in the field had swollen until it was spherical.   At this time two cells in the field had hemolyzed.   *13 minutes:* About 70 per cent of the cells in the field had hemolyzed, the rest were swollen but showed no trace of hemolysis. *16 minutes:* There were only two unhemolyzed cells left in the field.   *16½ minutes:* The last cell had hemolyzed.   The control cultures kept in the dark showed no · hemolysis.

In this laboratory, during the last 3 years, many thousands of supra-vital preparations of blood cells have been made and studied.   Some of these have been exposed to light for a number of hours, but no striking or rapid degeneration of these smears, due to the action of light, has been noted.   These supravital preparations were made by placing a drop of blood on a cover-slip and dropping the cover, blood surface down, onto a clean slide coated with neutral red or neutral red and Janus green, and sealing the culture with vaseline.

There being the possibility that a degeneration due to the action of light did occur in such a preparation, but that it had been overlooked, a group of such flat smear preparations were made, the blood being drawn from the ear vein of a rabbit.   No dye was used on these slides.   At the same time, a group of similar preparations were made, the only difference being that instead of inverting and sealing the cover-glasses on the usual plane slides, they were inverted over the wells of hollow ground slides, giving preparations such as those used in the last experiment.   The edges of the cover-slips were sealed with Salvoline.   Of these two sets of slides, half of each set was

irradiated with light from the source previously described, and the other half was kept in the dark incubator as a control. Data from representative cultures from these sets are given in Tables I and II.

TABLE I.

Time Required for the Degeneration of Leucocytes and Erythrocytes in Hanging Drop Preparations of Whole Blood.

Culture number	In light		In dark (control)	
	Time	Condition of cells	Time	Condition of cells
	min.		*min.*	
1051	180	r.b.c. swollen, hemolyzed; p. markedly degenerate		
1052			360	r.b.c. not hemolyzed; no data on p.
1053			360	r.b.c. not hemolyzed; no data on p.
1057	70	r.b.c. hemolyzed; p. rounding up, edematous		
1058			190	r.b.c. normal; p. normal
1058 A			190	r.b.c. normal; p. normal
1059	100	r.b.c. hemolyzed; p. rounded up, degenerate		
1060	50	r.b.c. swollen; p. rounded up		
1061			75	r.b.c. normal; p. normal
1062	65	r.b.c. swollen; p. rounded up		
1063			190	r.b.c. normal; p. normal
1317	120	r.b.c. swollen; p. rounded up		
1318			160	r.b.c. normal; p. moving very slowly
1319			160	r.b.c. normal; p. moving very slowly
Average ..	100 min. or 1.7 hrs. r.b.c. swollen, hemolyzing p. swollen, hemolyzing		197 min. or 3.3 hrs. r.b.c. normal p. normal	

r.b.c.. . .red blood cells.

p.. . . . . .polymorphonuclear neutrophils.

Table I, Columns 2 and 3, shows that an average of 1.7 hours was required for the neutrophils present in the irradiated area of the hanging drop culture to round up. At this time, many of the cells showed a distinct hydropic degeneration. By this time also the red

TABLE II.

Time Required for the Degeneration of Leucocytes and Erythrocytes in Flat Smear Preparations of Whole Blood.

Culture number	In light		In dark (control)	
	Time	Condition of cells	Time	Condition of cells
	hrs.		*hrs.*	
1025	6.5	r.b.c. normal; p. moving slowly		
1026			6.5	r.b.c. normal; p. moving slowly
1063	7.0	r.b.c. practically normal; few rounded up; majority still motile		
1064			7.0	r.b.c. in slightly better condition than in No. 1063; p. as in No. 1063
1450	4.0	r.b.c. normal; many p. rounded up; some still motile		
1451			4.0	r.b.c. as in No. 1450; p. normal
1452			4.0	r.b.c. as in No. 1450; p. normal
1453	6.0	r.b.c. slightly shrunken; no gross hemolysis; p. slowly motile		
1454			6.0	r.b.c. normal; p. normal
1455			6.0	r.b.c. normal; p. moving slowly
1456			6.0	r.b.c. normal; p. moving slowly
1457	11.0	Some of r.b.c. hemolyzed, some swollen; hemolysis slight; p. rounded up		
1458			11.0	r.b.c. as in No. 1457; p. as in No. 1457
1459			11.0	r.b.c. as in No. 1457; p. as in No. 1457.
Average.....6.9		r.b.c. normal or slightly degenerated p. slowly motile; some rounded up	6.9	r.b.c. slightly less degenerated p. slightly less degenerated

r.b.c...red blood cells.

p......polymorphonuclear neutrophils.

cells within the illuminated area were badly swollen and showed definite hemolysis, and in almost all cases free red cell pigment could be seen in solution in the culture medium.

Columns 4 and 5 of Table I show the results from similar cultures kept in the dark as controls. At the end of an even longer average time, 3.3 hours, there was no sign of a rounding up or of hydropic degeneration of the neutrophils of these cultures, nor was there any other sign of their degeneration. Further, even when the slides were examined after 4 hours, there was no sign of hemolysis of the red cells, except of those few cells injured in the preparation of the culture.

On the other hand, as shown in Columns 4 and 5 of Table II, at the end of 6.9 hours the neutrophils in the flat preparations kept in the dark, were moving around, though rather slowly, and the red cells showed only a very slight degeneration. The neutrophils in the flat preparations exposed to light were also still moving around, though very slowly, generally a little more slowly than in the control, while the red cells showed only slightly more degeneration than had been shown by those in the flat slide cultures held in the dark.

It seemed legitimate to conclude that this striking difference in the rates of degeneration of the blood cells on flat slides and in hanging drops, when irradiated, was due to the influence of some factor essential for the degeneration of both red and white blood cells when subjected to irradiation. In considering what this factor might be, there were three possibilities: (1) differences in evaporation from the two types of cultures, (2) differences in the thickness of the irradiated blood film, or (3) differences in the amount of air with which the culture was in contact.

An examination of the first of these, *i.e.* the influence of evaporation, showed that in the flat slide preparations the only marked evaporation which occurred was that which took place within the few seconds before the culture was sealed. On the hollow ground slide, however, in addition to this evaporation, there was also the evaporation from the hanging drop necessary to saturate the air in the hollow chamber of the slide. This chamber was found to have a volume of 0.25 cc., whereas the volume of the hanging drop of blood used, as measured from a volumetric pipette, was found to be about 0.005 cc. The saturation point of air for water vapor at 39° is about 0.00005 gm.

/cc., therefore 0.0000125 gm. of water vapor from the hanging drop would be required, to saturate the air in the well of the hollow slide. This represents an evaporation of about 0.25 per cent of the total volume of the drop. It is obvious that this additional evaporation is too small to constitute the factor responsible for the difference in the behavior of the two types of cultures under the action of light.

In considering the second possibility, that the thickness of the layer of red blood cells might constitute the essential difference in the two types of preparations, it was obvious that the hemolysis of the red cells and the degeneration of the white cells might be dependent on the greater absorption of light carried on by a relatively thick layer of cells, as in the hanging drop preparations. It might not occur at all, or to a very slight degree in films one cell thick, as in the flat slide preparations. This hypothesis was tested out by making a diluted suspension of red cells in unbuffered Locke solution, and irradiating hanging drops of these, as previously described. By this technique, films were obtained which were one cell thick, and which consequently had the same magnitude of light absorption as did the films on the flat slides. The concentration of serum in these cultures was lower than the concentration in the cultures of whole blood, but it has already been shown that the hemolysis of the red cells under the action of light, was not dependent on the presence or absence of such concentrations of serum (page 672). In these preparations, as in the hanging drops of undiluted blood, the hemolysis of the cells was striking, and showed conclusively that the essential difference between the behavior of the flat slide preparations and the hanging drop preparations of whole blood was not due to the thickness of the layer of blood cells.

On the other hand, while, owing to the marked reducing powers of the freshly drawn blood itself, the blood on the flat slide was under strongly anaerobic conditions within a very few minutes from the sealing of the culture, the hanging drop culture remained in contact with about 50 times its own volume of air. It seems logical to assume that this was the difference responsible for the divergent behavior of the two types of preparations when subjected to irradiation.

DISCUSSION.

The degeneration, or hemolysis, of the red cells under the action of light, as described in this article, accords with the results of previous workers, who have found that light does exert a marked hemolytic effect on red blood cells. In particular it confirms the work of Hasselbalch who found that this hemolytic action was caused by ultraviolet light, and also by light wave-lengths extending at least a short distance down into the visual spectrum. At the same time it extends Hasselbalch's work in showing that the rays active in causing this degeneration are distributed throughout the visual spectrum, lying in each of the three following zones: (a) $430\mu\mu$–$550\mu\mu$; infra-red; (b) $475\mu\mu$–$630\mu\mu$; $690\mu\mu$–infra-red; (c) $600\mu\mu$–infra-red.

In considering the relative activity of the blue, green and the red regions of the spectrum in causing this degeneration, it should be noted that the green filter transmitted about 23 per cent of the light reaching it, while the blue and red transmitted only about 5 per cent and 6.6 per cent respectively. The time required to produce hemolysis by the action of light passing through any one of these three filters was, within the limits of error, the same. This fact appears to be open to two possible interpretations: (1) that through chance, the activity of the spectral zone transmitted by each of these filters, times its total transmission, is practically identical for the whole series of three filters, or (2) that within the range of light intensities used, different intensities produced little change in the period of time required to cause hemolysis of the red cells. That the second condition prevailed was shown by irradiation of the cultures through a neutral 5 per cent filter. With such a filter, the time required to produce hemolysis of the red cells was practically the same as when no filter, or any one of the three above described, was used.

If a comparison is made between the process of degeneration of the neutrophils under the action of light, as previously described (4), and the process of degeneration of the red cells, the similarity is evident. Both types of cells swelled to a marked degree. In the neutrophils this swelling often occurred to such a degree as to rupture the cells, and in the red cells it became so great as to result in an extrusion of cell contents (pigment), although this extrusion did not

occur through a gross rupture of the cell wall.   It will be noted also, that the terminal stage of the degeneration in the neutrophils was often one of cell coagulation.   As has been stated, in some instances the hemolysis of the red cells was incomplete, the cells assuming an opaque, coagulated appearance after having lost only a part of their pigment.   It would seem that light had produced some degree of coagulation of the cells before all of the hemoglobin could escape, thus producing a condition in the red cells paralleling the terminal coagulation of the neutrophils.

One very striking point in regard to these experiments was the interrelation of the degeneration time of the red cells and of the neutrophils.   During the whole process of the degeneration of the red cells, up to the time when the release of hemoglobin began, there was generally little or no change in the neutrophils.   However, within a short time after hemoglobin began to appear in the surrounding medium, the neutrophils rapidly ceased moving and rounded up.   Following this, the degeneration of the white cells was rapid, and often within 10 or 15 minutes the neutrophils were showing a marked hydropic degeneration.

Inasmuch as the red cells are heavily pigmented, whereas the white cells are practically achromatic, it appears natural that the former should, because of their greater light absorption, degenerate far sooner than the latter.   That this is the case has been shown in these experiments, in which the red cells generally showed a marked hydropic degeneration before the white cells were visibly affected.   At the same time it is possibly more than a coincidence that the degeneration of the white cells was often first noted immediately following the escape of hemoglobin from the red cells.   The question is naturally raised as to what rôle, if any, the substances liberated from the red cells in such a degeneration may play in causing the degeneration of the white cells.

One rather striking point brought out by these experiments is the difference in the manner of hemolysis of the cells in whole blood and in blood diluted with unbuffered Locke solution.   Upon irradiation of either, there was a gradual swelling of the cells.   Following this swelling, in whole blood, the liberation of pigment often occurred with almost explosive rapidity, every cell in the field undergoing complete

hemolysis in just a minute or so. In cases where this rapid hemolysis was not found, there was apparently a gradual loss of hemoglobin from all of the cells simultaneously. This process sometimes required as long as several hours. In dilute cell suspensions however, after the cells had become spherical, the cells all hemolyzed one or two at a time, as described by Dreyer and Hanssen (3). While this difference was sharply defined, the data are at present too incomplete to allow any conclusion as to the factors responsible for it.

In considering the influence of serum on the hemolytic action of light on red blood cells, the hemolysis of the cells occurred either when serum was present only in traces, or in large amounts. While it has been impractical to study the action of light on cells freed from the last trace of serum, it would appear that the hemolysis is not dependent on the presence or absence of serum, although the serum may serve to retard the reaction.

In regard to the comparative behavior of the flat preparations and the hanging drop preparations of whole blood, when irradiated, the factors of evaporation, of the thickness of the blood film, and the concentration of the blood serum have been eliminated. The data indicate that the essential difference between the two types of preparations has to do with the amount of air with which the culture is in contact. In the hanging drop preparations the blood is in equilibrium with about 50 times its own volume of air, whereas, in the flat preparations, the blood is so sealed that it has no contact with the air.

While this conclusion supports the belief of Busck (1), that oxygen plays some rôle in the hemolysis of red cells under the action of light, it does not agree with the conclusions of Hasselbalch (5), who found that it occurred in the absence of air. Because of the many points of variation in the technique employed by Hasselbalch and that employed in this work, further data will be necessary before an accurate comparison of results can be made.

SUMMARY.

1. It was found that when hanging drops of whole blood, drawn from a rabbit, were subjected to irradiation from certain light sources, a striking degeneration of the white and of the red cells occurred. In this degeneration of the red cells there was (a) a preliminary period

of 15–30 minutes during which no effect was noted; (*b*) following this, there was a period during which the cells swelled and became first almost biplanar, then biconvex, and finally spherical. After a spherical shape had been assumed, the liberation of blood pigment from the cells began. This process progressed with varied rapidity, sometimes being very slow, and being incomplete; sometimes being of almost explosive rapidity. This liberation was not accomplished in either case through a gross rupture of the cells, but through the agency of some submicroscopic change produced either in the cell contents, the cell wall, or both. Following this liberation of pigment, in some cultures the red cells showed signs of coagulation, as did the white cells. In other cultures, however, the red cells were reduced to achromatic shadows.

2. When whole blood was diluted with unbuffered Locke solution and then irradiated in hanging drop preparations, the erythrocytes swelled, as in whole blood, but then, instead of an almost instantaneous hemolysis of every cell present, or a slow liberation of hemoglobin from each individual cell, all the red cells hemolyzed, one or two at a time, the hemolyzed cells being left as achromatic shadows.

3. This hemolysis of the red cells, as in the case of the degeneration of the white cells, occurred upon irradiation of the culture with white light, or with light lying in each of the three spectral zones of the visual spectrum, defined by Wratten filters Nos. 45, 58 and 29 respectively, as follows: (*a*) $430\mu\mu$–$550\mu\mu$; infra-red; (*b*) $475\mu\mu$–$630\mu\mu$; $690\mu\mu$–infra-red; (*c*) $600\mu\mu$–infra-red.

4. Within the range of intensities of light employed, there was little or no difference in the rate at which light of these three regions acted on the red cells. Further, this rate was the same as was the rate when the red cells were irradiated with no colored filter interposed in the optical path, or when a 5 per cent total transmission neutral filter was interposed.

5. It was shown that this degeneration of the red cells under the action of light was not dependent on the presence or absence of serum, with the possible exception of that trace which might have been adherent to the red cells even after repeated washing.

6. The white cells in the culture generally showed no changes until after traces of red cell pigment could be seen free in the surrounding medium.

7. On the other hand, no neutrophils were observed moving around for longer than a few minutes after the liberation of red cell pigment had occurred.

8. The liberation of substances from the red blood cells, as a result of irradiation of such preparations, may play some major rôle in the degeneration of irradiated white cells.

I wish to express my appreciation to Dr. R. S. Cunningham, of the Department of Anatomy, under whose supervision this work has been carried on, to Drs. W. E. Garrey and C. E. King, of the Department of Physiology, and to Dr. G. E. Cullen, of the Department of Biochemistry, for their helpful suggestions and aid.

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STUDIES UPON THE EFFECT OF LIGHT ON BLOOD AND TISSUE CELLS.

III. The Action of Light on Fibroblasts in Vitro.

By W. R. EARLE.*

(*From the Department of Anatomy, Vanderbilt University School of Medicine, Nashville.*)

Plate 17.

INTRODUCTION.

In the course of a series of observations on the effect produced on leucocytes and erythrocytes by irradiation *in vitro* (1), it was found that these cells underwent an extreme and rapid degeneration when exposed to light of various regions of the visual spectrum. The data obtained were such as to suggest that substances liberated from the erythrocytes, during their degeneration under the influence of light, played some rôle in the degeneration of the leucocytes when exposed to light. For the further analysis of this problem it seemed essential to obtain the leucocytes free from erythrocytes. Inasmuch as attempts to do this were unsuccessful, it was decided to try the fibroblast, which cell was found to be fairly satisfactory. Further work, detailed in this article, has indicated that the presence of the red blood cells does play a major rôle in degeneration of the fibroblasts under the action of light of the visual spectrum.

Kiaer (3), in 1925, studied the action of light from a Kromayer quartz mercury vapor lamp on pure cultures of fibroblasts. He found that the light from this source exerted a definite impeding action on the growth of the fibroblasts in cul-

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ture, and that an exposure of from 30–60 minutes resulted in the death of the cells. Light from this source is very rich in ultra-violet wave-lengths and as these are known to produce striking biological effects, Kiaer's data cannot be considered as necessarily applying to the longer wave-lengths of light lying within the range of the visual spectrum, with which the previous articles of this series (1, 2) were concerned.

Material and Methods.

The fibroblasts used in this investigation were obtained from the hearts of embryo chicks of from 6–12 days incubation. In obtaining the tissue, the chick was removed from the egg and washed in Tyrode solution (pH 7.4). The heart was then removed and cut up in the same solution, using a pair of iridectomy scissors for this work. Fragments of the tissue were then transferred to cover-glasses by means of a platinum loop, and there covered with a drop of Tyrode solution (pH 7.4). The cover-glass was then inverted over the well of a hollow ground slide, and its edges sealed with Salvoline. This whole process was carried out aseptically in very dim light. The cultures were immediately set in a water-jacketed incubator in the dark and kept there at 38° until the fibroblasts had moved out on the cover-slip in a sheet of some size. This required from 10–60 hours. At the end of this time the cultures were divided into two groups; one group was irradiated, while the other group was kept in the dark incubator as a control.

The cultures which were to be irradiated were all given a careful examination at the beginning of irradiation and were discarded at once if any trace of cellular degeneration was present. Inasmuch as the least examination of the controls would have vitiated the experiment, these could not be given a preliminary examination.

The light source and optical system used in this work were the same as those used in the previous work (1). Spectrograms of this light source, and of this light source with the special filters described later, have already been shown (Earle (1), spectra 4, 5, 6 and 7, Fig. 4). In all of these experiments, irradiation and microscopic examination of the cultures were carried out simultaneously.

EXPERIMENTAL.

In the preliminary work on this problem, an attempt was made to irradiate the fibroblasts under conditions as similar as possible to those under which the white cells degenerated. The factors which had to be taken into consideration were, (1) that the white cells themselves were present (2) that red cells were present in varying numbers, (3) that even in cultures of cells in saline, at least traces of serum were present and (4) that air was present in large volumes compared with the actual volume of the explant.

In order to secure cultures of fibroblasts in which these conditions prevailed, red blood cells were obtained by puncture, with a capillary pipette, of one of the extra embryonic blood vessels of an embryo chick. These red cells were washed with three changes of Tyrode solution having a pH of 7.4. Cultures were then made using tissue from the heart of the same chick. These cultures were inoculated, as described above, except that a few of the washed red blood cells were also added. Some of these cultures were irradiated while the others were kept in the dark as controls.

An examination of these irradiated cultures showed conclusively that under these conditions the light did cause an extreme degeneration of the fibroblasts. Beginning at about 15–30 minutes after the irradiation was started, the refractive index of the fibroblasts showed a gradual but distinct increase. Normally the fibroblasts in culture have such a low refractive index as to be seen only with difficulty, while the nuclei are exceedingly difficult and often impossible to discern. In these irradiated cultures, however, the refractive index of the fibroblasts increased so greatly that the cells and all of their processes could be outlined with ease. At the same time, the nuclei also became sharply defined.

After the lapse of from 30–140 minutes, vacuoles of low refractive index began to appear throughout the cytoplasm of the cells. These vacuoles did not appear to originate in any single region of the cells, nor did they generally seem to begin as minute sharply defined droplets, increasing in size. Rather, in some region where the cytoplasm had appeared homogeneous, there appeared a small area with a very slightly lower refractive index than the surrounding region, but at first almost indistinguishable from it. The optical distinction between this area and the surrounding region increased rapidly, however, until the droplet appeared as a clear area of low refractive index, a "punched out" looking area in the cell, surrounded on all sides by the more highly refractive cytoplasm. These vacuoles were of all sizes and occurred in different numbers in different cells. When irradiation was continued for 3 or 4 hours, practically every cell in the culture was densely crowded with them. Upon staining the cultures supravitally with neutral red, they took up some of the dye, and were colored orange-red by it. They showed no reaction with Sudan III,

or with osmic acid. From these staining reactions and from their low refractive index, it would seem that their chief content was probably water. About the time the vacuoles began to appear, the cells often showed some tendency to round up. If irradiation was continued until the cells were crowded with vacuoles, this rounding up was almost constant in its appearance, and often occurred to a very marked degree (Fig. 1).

A modified form of this degeneration was frequently seen in these cultures. Instead of the formation of discrete vacuoles in the cytoplasm of the cells, the viscosity of the cytoplasm showed a great decrease, as indicated by the marked increase in the amplitude of brownian movement of intracellular particles. The cells began swelling very rapidly, and, as a result, became almost spherical. None of these cells was ever seen to burst as a result of this swelling.

In the unirradiated fibroblasts in tissue culture, there were generally a few highly refractive droplets staining with Sudan III or with osmic acid, and obviously of a lipoid nature. At the end of several hours of irradiation, these droplets were often perceptibly increased, both in size and in number. The dominant features in this degeneration, however, were the change in the refractive index of the cell and the formation of vacuoles throughout its cytoplasm.

The whole process of degeneration as above described, often took place within 1 hour, and never required longer than 3. This degeneration was remarkably constant in its appearance, although in some cultures it appeared in the modified form previously described. In this modified form the changes seen appeared to be identical with those seen in the polymorphonuclear neutrophils under the action of light.

The protocols from a representative culture of this series, and its controls are here shown as Experiment 1.

Experiment 1.

Culture 801.—This culture was planted from the heart of an 11 day embryo chick. *16 hours after inoculation:* Irradiation was begun. At this time the fibroblasts had grown out in a sheet. The cells appeared normal; their refractive index was low, and a few fine highly refractive droplets were scattered through them. There was no sign of vacuoles in any of the cells. *40 minutes later:* A marked in-

crease in the refractive index of the cells had occurred; details of cell boundaries and nuclei could be made out. At this time some of the cells showed a single vacuole from one to four times as large as the nucleus. *100 minutes:* Many cells showed definite vacuoles, and quite a few were filled with them. In most of the cells the vacuoles were unmistakably of cytoplasmic origin, some of them originating far out in the pseudopodia. *300 minutes:* Practically all of the cells were filled with vacuoles. In this respect there had been little change in the last 150

TABLE I.

Experiment 2.

Time Required for the Degeneration of Fibroblasts in Vitro When Irradiated by Light Transmitted by Various Filters.

Culture number	Age of chick	Organ from which culture was taken	Age of culture	Time required for degeneration			
				No filter Transmission 100 per cent	Filter No. 45 (blue)	Filter No. 58 (green)	Filter No. 29 (red)
	days		*hrs.*	*min.*	*min.*	*min.*	*min.*
829	8	Heart	18			120	
831	8	Heart	27		630		
832	8	Heart	39				300
853	12	Heart	18	195			
854	12	Heart	About 26	160			
858	12	Heart	39				780
859	12	Heart	51				570
860	12	Heart	60	165			
897	9	Heart	35			130	
921	8	Intestine	14	145			
922	8	Intestine	About 16			90	
931	8	Intestine	About 21	160			
936	8	Intestine	18	100			
937	8	Intestine	20			70	
1579	8	Heart	About 16		600		
Average..............................				158	615	102	550

minutes. The cells showed distinct signs of rounding up. The preparation was photographed at this time (Fig. 1).

Cultures 805 and 807.—Control cultures. Kept in the dark incubator. *21 hours after inoculation:* The fibroblasts appeared normal. They were of low refractive index, and were very difficult to see. There were only a few fine droplets in the cells, and no vacuoles were present.

The attempt was next made to see if, as in the case of the leucocytes, the degeneration of the fibroblasts was caused by wave-lengths of light

lying throughout the range of the visible spectrum. Accordingly, cultures were inoculated and, as in the case of the experiments on the white blood cells (1), were irradiated through Wratten filters Nos. 45, 58 and 29. The time required for the fibroblasts in each culture to undergo degeneration when that culture was irradiated by light transmitted by any one of these three filters is shown in Table I. This table also shows the time required for the degeneration of control cultures irradiated with the same light source but with no Wratten filter inserted in the optical path. Although not presented in this table, other control cultures, inoculated as above but kept in the dark, remained normal.

In preparing this table, the degeneration time for any culture was arbitrarily defined as the time required for the appearance of a marked increase in the refractive index of a majority of the fibroblasts in the microscopic field, accompanied either by the appearance of many vacuoles, in the cytoplasm of the cells, or by a marked tendency for the cells to round up and to show a greatly increased amplitude of brownian movement of their intracellular particles. It may be seen from this table that wave-lengths of light transmitted by any one of the three filters used caused the typical degeneration of the fibroblasts.

The next question considered was the possible mechanism of this action of light on the fibroblasts. In this consideration, three possibilities arose. The degeneration of the fibroblasts under irradiation might have been due to, (1) an intrinsic action of light on these cells, or (2) an intrinsic action of light upon the red cells with the formation of toxic substances, which in turn produced a degeneration of the fibroblasts, or (3) to a sensitization of the fibroblasts by some substance liberated from the red cells, with a subsequent action of light on the sensitized fibroblasts.

In order to determine whether the red blood cells did play a rôle in the degeneration of fibroblasts under the influence of light, cultures were prepared as before, except that this time no red cells were added to the culture. These cultures were then placed in two groups, one group being kept in the dark as a control, while the other was irradiated. A further control was kept on these cultures by irradiating similar cultures containing fibroblasts and red cells. All the cultures containing red cells and exposed to the action of light showed a typical

degeneration of the fibroblasts. In one group of eight cultures irradiated, every culture showed a typical extreme degeneration in less than 3 hours. On the other hand, of five cultures, supposedly containing no red blood cells, one showed the typical degeneration only after irradiation for 13 hours. The other four showed only minor degenerative changes at the end of from 13–24½ hours of irradiation. These minor changes consisted of an increase in the refractive index of the cells and the rounding of some of the sharper protoplasmic processes. In these cultures there was no indication whatsoever of any swelling of the cells, of any increase in the brownian movement of intracellular particles, nor of any formation of vacuoles within the cytoplasm. This contrast, between the behavior of the cultures containing red cells and that of the cultures containing no red cells, was augmented by the fact that at the close of the irradiation of the cultures containing no red cells, even some of the controls which had been kept in the dark, were showing traces of degeneration. In every instance, however, this degeneration of the controls was markedly less than that in the cultures kept in the light.

A protocol of a representative culture of this series is presented below as Experiment 3.

Experiment 3.

Culture 823.—Inoculated from the heart of an 11 day embryo chick. *24 hours after inoculation:* Irradiation was begun; the cells appeared normal at the time although they contained a good many fine droplets of fatty material. *100 minutes later:* There had been no change except a very slight increase in the refractive index of the cells. *270 minutes:* There had been no change and the cells appeared normal. *15 hours:* The cells showed a somewhat higher refractive index than was normal, and their nuclei were sharply defined. They were not rounding up and showed no vacuoles or swelling. *17 hours:* There had been little change. A representative cell was photographed at this time (Fig. 3).

DISCUSSION.

There was a definite similarity between the type of degeneration seen in the fibroblasts and that observed in the erythrocytes and in the leucocytes. The similarity between that shown by the fibroblasts and by the neutrophils was particularly striking. No formation of discrete vacuoles of large size has been observed in the neutrophils,

probably owing to their more fluid cytoplasm and its consequent more general liquefaction. It should be noted, however, that sometimes vacuole formation in the fibroblasts was so slight that no vacuoles could be made out in the unstained cells, the cytoplasm undergoing just such a liquefaction as occurred in the neutrophils. This liquefaction was indicated by a tremendously increased amplitude of brownian movement of particles within the cells. When this occurred the cells rounded up, became almost spherical, and swelled rapidly. Time and again, fibroblasts were seen in this state, and if the complete process of degeneration of the individual cells had not been followed, they would have been indistinguishable from the swollen polymorphonuclear neutrophils seen in the cultures of blood which had degenerated under the influence of light (Earle (1), Fig. 1).

It was found, as in the case of the leucocytes and the erythrocytes, that light of wave-lengths lying within the three zones (a) $430\mu\mu$–$550\mu\mu$; infra-red; (b) $475\mu\mu$–$630\mu\mu$; $690\mu\mu$–infra-red; (c) $600\mu\mu$–infra-red, was active in causing this degeneration of the fibroblasts. The relative activity of these three zones for the erythrocytes is apparently not the same as for the fibroblasts, for the intensities of light employed. The light transmitted by each of these three filters caused an hemolysis of the red cells within practically the same time, and within the same time that was required by white light of even greater intensity. It was found, however, that when either the blue filter No. 45 (less than 5 per cent total transmission) or the red filter No. 29 (total transmission about 6.6 per cent) was placed in the beam of light irradiating the fibroblasts, the time required for the degeneration of the cells was much greater. A similar, though a less striking phenomenon was also noted in the cultures of leucocytes previously irradiated (1). This difference between the leucocytes and the fibroblasts on the one hand, and the erythrocytes on the other, is most interesting, although at present no explanation for it is offered.

Another interesting point brought out by these data is that when irradiated through the green filter, the fibroblasts consistently seemed to show a more rapid degeneration than when irradiated with white light (100 per cent transmission). This was also found true for the cultures of irradiated leucocytes (1). Before any definite conclusions are drawn on this point, however, these data should be verified and extended by means of a more accurate quantitative technique.

It was found that the fibroblasts, in cultures to which no red cells had been added, had degenerated much less at the close of from 13–24 hours of irradiation than they had at the end of from 2–3 hours irradiation when red cells were present.   This observation indicates that the red cells played some major rôle in the degeneration of the fibroblasts. In these cultures, supposedly containing no red cells, the tissue was carefully washed, and red cells were certainly very few in number. However, a very few red cells were seen in the culture which showed the typical degeneration at the end of 12 hours.   In the other cultures it is quite possible that either isolated red blood cells or traces of the substances liberated from disintegrated red cells were present. For this reason, it is impossible to say that even the traces of degeneration seen in these cultures were due to an action of light directly on the fibroblasts alone.   Certainly any action of light of the visual spectrum on the fibroblasts alone was exceedingly slight, its major influence being conditioned by the presence or absence of red blood cells.

Whether the presence of the red cells is also essential to the degeneration of the leucocytes under the action of light has not as yet been determined.   There are, however, several facts which indicate that it plays some rôle.   In tissue cultures of leucocytes it was noted that, for any one light source, there was a marked variation in the time required for the degeneration in different cultures of any one series. In these cultures, there was also a marked variation in the number of red blood cells present.   Furthermore, in hanging drops of whole blood, the degeneration of the leucocytes rarely occurred before there were marked signs of degeneration of the erythrocytes, and it was never delayed more than a few minutes after the hemolysis of the erythrocytes had begun.

SUMMARY.

1. In the presence of autogenous red blood cells, fibroblasts, grown *in vitro* from the heart and intestine of embryo chicks of from 6–12 days incubation, underwent a rapid degeneration when exposed to light of the visual spectrum.

2. In this degeneration, the cells showed a marked increase in refractive index, and a massive formation of colorless vacuoles of low refractive index.   These vacuoles gave no reaction with osmic acid

or Sudan III, but took up neutral red.   Later in the degeneration the cells showed marked signs of rounding up and of coagulation.

In some cases this degeneration process was modified in such a manner that, instead of the formation of vacuoles, the whole cytoplasm became much less viscid and the cells swelled greatly and became spherical.

3. The similarity of this degeneration to that shown by the erythrocytes, and more particularly, to that shown by the polymorphonuclear neutrophils, under the action of light, is pointed out.

4. Degeneration of the fibroblasts occurred when the cells were irradiated by light of any one of the following wave-length zones: (a) $430\mu\mu$–$550\mu\mu$; infra-red; (b) $475\mu\mu$–$630\mu\mu$; $690\mu\mu$–infra-red; (c) $600\mu\mu$–infra-red.

5. This degeneration was much slower for the cells irradiated through the blue or red filters of less than 5 per cent, and less than 6.6 per cent, respective total transmissions.   When irradiated through the green filter of 23 per cent total transmission it was much more rapid, and even more rapid than for cultures irradiated with white light of 100 per cent total transmission.   These data were obtained on a short series of cultures and must be considered only as suggestive.

6. In the apparent absence of red cells, the fibroblasts underwent only a slight degree of degeneration after irradiation lasting from 13–$24\frac{1}{2}$ hours.   It is possible that even this minor degeneration may depend upon the presence of traces of disintegration products of red cells in the culture.   Much more advanced degenerative processes were obtained, when many red cells were present, after only 3 hours irradiation.

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EXPLANATION OF PLATE 17.

Fig. 1. Culture 801, × 1000 (see text, page 687).   Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated with erythrocytes present in the culture.   Irradiated for 5 hours.   Photographed so that half of the photographic plate was given a longer exposure than the other half.   The fibroblasts show a striking increase of refractive index.   There was a marked rounding up of the cells and the cytoplasm was filled with vacuoles.

Fig. 2. Culture 820, × 1000.   Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated in culture, no erythrocytes being present.   Photographed 52 hours after inoculation of the culture and after 13 hours of irradiation. Note that while the refractive index of the cells is somewhat greater than normal, and that their cytoplasm is somewhat more granular than normal, there is no sign of rounding up of the cells, nor are there any vacuoles visible in the cytoplasm.

Fig. 3. Culture 823, × 1000.   Fibroblasts taken from the heart of an 11 day chick embryo, and inoculated in culture, no erythrocytes being present.   Photographed 40 hours after inoculation and after 17 hours of irradiation.   Note that there is some tendency for the cell to round up; its nucleus is clearly outlined, and the refractive index of the cell is slightly increased.   There is far less degeneration than was shown in Culture 801, Fig. 1, however.
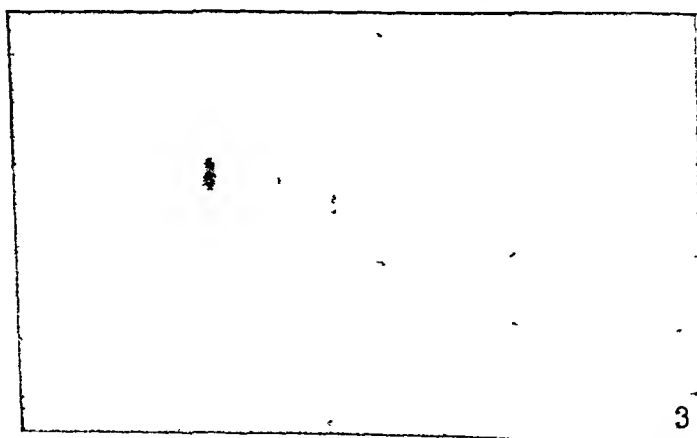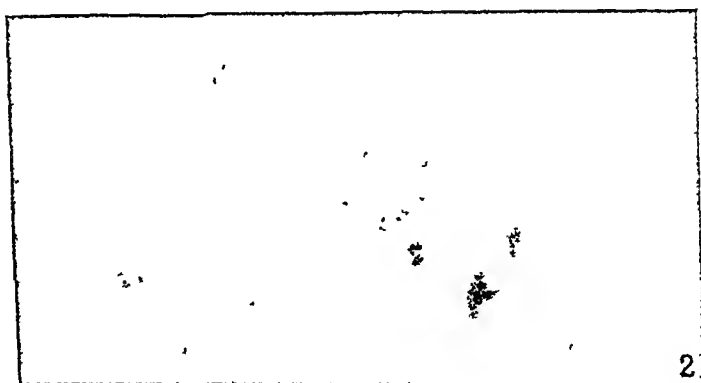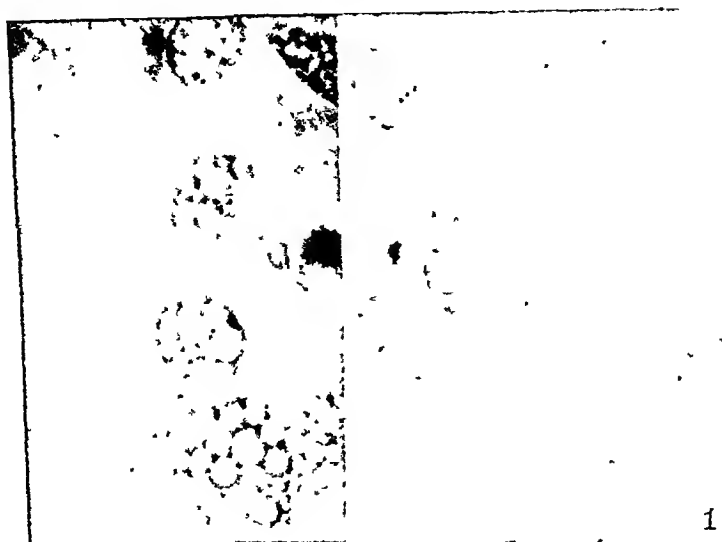
EXPERIMENTAL PNEUMONIA IN GUINEA PIGS.

I. THE EFFECT OF CERTAIN TOXIC AUTOLYSATES OF PNEUMOCOCCI.

BY JULIA T. PARKER AND ALWIN M. PAPPENHEIMER, M.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)

PLATES 18 AND 19.

Much work has already been carried out on the experimental production of pneumococcus pneumonia in animals. Important publications on the subject are those of Wadsworth (1) on the production of pneumonia in rabbits; of Lamar and Meltzer (2) and Meltzer and Wollstein (3) in dogs; of Cecil and Blake (4) in monkeys; and of Stillman and Stillman and Branch in mice (5–7).

It is logical to suppose that in order to produce pneumococcus pneumonia this organism must be made to grow in the lung. It has, however, been the experience of all workers that, in the case of the smaller laboratory animals—rabbits, guinea pigs, or mice—pneumococci will not infect the lung tissue save in exceptional instances, no matter how they are injected. Thus Wadsworth found that rabbits injected intratracheally with virulent or non-virulent pneumococci either failed to become infected or died with pneumococcus septicemia, but without pneumonic lesions. Stillman had the same experience in his attempt to produce pneumonia in rabbits by the inhalation method. In the case of mice also Stillman found that virulent pneumococci, though inhaled into the lower respiratory tract, disappeared within a few hours without calling forth a pneumonic reaction. Even in alcohol-intoxicated mice in which the pneumococci persisted in the lung for a longer period than in unintoxicated mice and in which fatal septicemia often followed, localization with production of pneumonia was rarely observed.

Wadsworth's negative results in attempting to produce pneumonia

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in normal rabbits led him to repeat the experiments in systemically or locally predisposed rabbits. In this he was unsuccessful as was Stillman in similar experiments in mice. However, by provoking a partial immunity in rabbits, Wadsworth did succeed in regularly causing pneumonic lesions and recently Stillman has also succeeded in producing lobar pneumonia in partially immunized alcoholized mice exposed to an atmosphere of virulent pneumococci.

The obvious conclusion that emerges from this work on the production of pneumococcus pneumonia in the smaller laboratory animals is that under ordinary conditions, the pneumococci will not grow in the lungs of these animals and without such growth pneumonia is not produced. If this be true, one may imagine that in pneumococcus pneumonia in animals, and in man, there is a hypothetical substance or substances, perhaps produced in the growth or autolysis of the pneumococci, which by its action upon the lung tissues or fluids enables the pneumococcus to establish itself and to multiply, and in so doing to cause pneumonia.

In a previous communication (8) it was shown that Berkefeld filtrates of certain anaerobically produced autolysates of Pneumococcus I or Pneumococcus II cause necrosis when injected intracutaneously into guinea pigs. In the course of this work it was discovered that these autolysates when injected intratracheally were highly toxic, and that when introduced in this manner together with living pneumococci, the growth of the organisms in the lung became established. From these observations it seemed possible that such toxic filtrates might contain the hypothetical poisonous substances referred to above, and this report gives the experiments which were conducted in the study of this problem.

The experiments here reported refer (1) to the effect of toxic autolysate alone upon the lungs of guinea pigs, (2) to the effect of diluted toxin added to living pneumococci, in establishing the growth of the organism in the lungs. In this second group of experiments, controls were given of equivalent doses of (a) toxic autolysate alone, and (b) living pneumococci without autolysate.

We will discuss the biological and immunological properties of the lung-toxic substance or substances and compare these properties with those of the necrotizing poison in the autolysates in a later publication.

Intratracheal Injection of Toxic Autolysates Alone.

Preparation of Toxic Autolysates.—The method used in preparing the toxic autolysates was the same as that previously described (8).

Virulent Pneumococcus Type I or Type II was grown on double strength veal infusion broth containing 4 per cent Witte peptone. After 18 to 24 hours growth, and before any appreciable autolysis had taken place, the broth cultures were chilled and then centrifuged at high speed. After centrifuging the tubes were chilled, the supernatant fluid pipetted off carefully, and the pneumococci taken up in a quantity of freshly boiled and chilled broth equal to that of the combined sediment and supernatant fluid remaining. For the pneumococcus sediment from 100 cc. of broth culture, there should be in all approximately 1.5 cc. of fluid. The pH of the pneumococcus suspensions was brought to 7 or 7.2 and cultures made to be sure that only pneumococci were present in the preparation. Sometimes 0.25 per cent phenol was added to the preparations. The suspensions were distributed into narrow test-tubes, which were then chilled for at least 30 minutes. After this, any bubbles present on the surface of the suspensions were gotten rid of with a hot platinum loop and heavy vaseline seals added to all of the tubes. The tubes were left at room temperature in the dark at 22-24°C. for 2 to 5 days and then placed in the ice box. Before filtering, the autolysates were centrifuged, iced, the seals opened, and the clear supernatant fluid passed through a well iced Berkefeld filter. This filtrate contains the poison which is highly toxic when injected intratracheally. The filtrate was kept in the ice box under vaseline seal until used. It was necessary to keep the preparations chilled when they were exposed to the air, otherwise they became oxidized and the toxicity disappeared.

(A) Results of Intratracheal Inoculation of Undiluted Toxic Autolysates Alone.

It was found that strong necrotizing filtrates invariably produced marked symptoms followed by death when injected intratracheally in 0.2 cc. amounts in guinea pigs of from 180 to 300 gm. weight. The toxicity for the skin and for the lungs appeared to run parallel; that is, a filtrate which produced marked necrosis when injected intracutaneously was also extremely toxic when injected intratracheally into guinea pigs, while a filtrate of weak necrotizing activity caused only slight symptoms when injected intratracheally and the guinea pigs usually survived. Although there were individual differences in the reaction of guinea pigs even of the same weight after the injection of

a strong toxin, 0.2 cc. of the undiluted poison usually killed them in less than 3 hours, and, with three exceptions, in less than 24 hours. Guinea pigs almost invariably survived the intratracheal injection of strong toxin diluted 1 to 2 or 1 to 4 with broth.

1. Guinea Pigs Dying within 1 to 2 Hours after Injection.

Symptoms and Pathology—Within 5 minutes of the intratracheal injection of the toxic autolysates the guinea pigs showed marked dyspnea which continued until death.   In the pigs which died in less than 6 hours, tonic and clonic muscular contractions and a bloody serous nasal discharge were noted before death.   At autopsy, the lungs of the guinea pigs which died in less than 6 hours were greatly distended, very heavy, and mottled deep pink throughout.   The cut section of these lungs centrally was extremely wet, deep red, and apparently airless.

Histological Findings.—Eight guinea pigs dying within 1 to 2 hours after injection were studied.   All of these showed severe and definite pulmonary lesions: intense congestion, profuse alveolar hemorrhages, edema, stasis of leucocytes in the alveolar capillaries, and the presence of sparse numbers of emigrated leucocytes in the alveoli.   The peribronchial tissues also were edematous, and loosely infiltrated with wandering cells.   Fibrin was not conspicuous.

2. Guinea Pigs Surviving for 18 to 72 Hours after Injection.

In this group, $\frac{2}{3}$ to $\frac{3}{4}$ of both lungs was found at autopsy distended, heavy, deep red, and consolidated.   There was a varying amount of normal tissue at the margins of some of the lobes.   The cut surface of the red portions appeared completely solid.   This group included animals injected with weak autolysates or with strong autolysates diluted 1 to 2 or 1 to 4 with broth and killed with ether at varying intervals after the injection.   At autopsy, the appearance of the lung was similar to that described above save that the consolidated areas were concentrated in the hilum regions.   It should be emphasized that the color of the pneumonic areas in all the animals injected with toxic autolysate alone—and over 100 have been examined—always was deep red and never even tinged with gray.

Histological Findings.—Fourteen animals of this group were studied. All showed patchy but quite extensive areas of pneumonic consolidation. The alveolar exudate in the earlier stages consisted of well preserved polymorphonuclear leucocytes, numerous red blood cells, and occasional large mononuclears. Fibrin was not abundant. There was swelling of the alveolar cells, necrosis in some areas of the alveolar wall with occasional formation of hyaline membranes. Edema of the peribronchial tissues and alveoli was less marked than in animals dying in the first few hours and massive hemorrhages were not present.

In animals killed after the 2nd day there were still pronounced pneumonic lesions, the ductuli alveolares were dilated and inflamed, and small pneumonic patches were scattered through the tissues; edema was still quite marked.

On the 3rd day the lesions showed evidence of regression, leucocytes were less numerous and undergoing fragmentation and autolysis. There was swelling and active proliferation (mitoses) of alveolar epithelium and capillary endothelium. In one animal the peribronchial lymphoid tissue appeared hyperplastic.

Results from Intratracheal Injections of the Following Preparations in 0.2 Cc. Doses.

B. Equal parts of pneumococcus cultures and toxic autolysates (undiluted or diluted 1 to 2).

C. Equal parts of pneumococcus cultures and broth.

D. Equal parts of toxic autolysates undiluted or diluted 1 to 2.

In every experiment in which pneumococcus cultures and toxic autolysates were injected together, controls of the same quantity of the pneumococcus culture alone (C) and the same quantity of the toxin preparation alone (D) were always injected separately into other guinea pigs.

Pneumococcus Strains Used.—The strains of Types I and II of the pneumococcus used in this work were the same as those used previously. Both strains when injected intraperitoneally were very virulent for rabbits, mice, or guinea pigs.

In these experiments we used only those filtrates which would kill guinea pigs within 24 hours, in undiluted doses of 0.2 cc. when injected intratracheally.

Technical Procedure.—18 to 24 hour broth cultures of Pneumococcus I and Pneumococcus II were used in this work. When there was profuse growth, the culture was diluted with broth to obtain the slight clouding desired. The pneumococcus culture was kept in ice water throughout the experiment.

Immediately before inoculation the tube containing the toxic filtrate was chilled, the vaseline seal opened, and the filtrate pipetted into a narrow chilled tube. This tube was kept in ice water throughout the experiment. Just before the injection, 0.2 cc. of the pneumococcus culture and 0.2 cc. of the toxin were pipetted into another iced tube, mixed, and taken up into an iced syringe; 0.2 cc. of this mixture was then immediately injected intratracheally into a guinea pig of 180 to 300 gm. A fresh mixture was always prepared before each injection, and the same precautions as to chilling, etc., were carried out with both control preparations of pneumococcus cultures and broth (C) and of toxic autolysate and broth (D).

(B) Results From the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.

Forty-five guinea pigs were injected with mixtures of toxin and pneumococcus cultures. With one exception all died with pneumonic lesions and positive lung cultures. These forty-five guinea pigs may be conveniently grouped as follows:

1. 8 guinea pigs (18 per cent) which died in 6 hours or less.
2. 35 guinea pigs (77 per cent) which died in from 18 to 72 hours.
3. 2 guinea pigs (5 per cent) which survived longer than 3 days.

In this series are included a certain number of animals which were injected intraperitoneally 18 to 24 hours previous to the intratracheal injection of pneumococcus culture and autolysate with normal serum, either rabbit or horse, or with certain heterologous antipneumococcus horse sera. It will be shown in a later report that none of these sera had an appreciable effect upon the subsequent intoxication and infection caused by the intratracheal injections of mixtures of toxic autolysates and pneumococcus cultures.

1. Guinea Pigs Dying within 6 Hours after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysate.

These guinea pigs showed the same symptoms as those which had been injected with 0.2 cc. of a strong toxin alone. They were markedly dyspneic directly after the injection and continued in this condition

until death. At autopsy, the appearance of the lungs of these animals was also similar to that found in the guinea pigs which had been inoculated with toxin alone. Cultures from the lungs always gave a growth of the pneumococcus, and positive cultures were obtained from the heart blood in five of eight pigs. We believe that these animals were exceptionally susceptible to the toxin and probably would have died had 0.2 cc. of toxin diluted 1 to 2 been inoculated without pneumococcus culture.

Histological Findings.—Five animals (8-68, 7-59, 10-13, 43-74, and 7-07) of this group were studied histologically. In the two which died after $1\frac{1}{4}$ and $1\frac{1}{2}$ hours respectively, there had already developed loose areas of consolidation about the large bronchi in the hilus region. In these pneumonic patches the alveoli contained an exudate with fairly abundant polymorphonuclears; red corpuscles were very numerous; there was a slight amount of delicate fibrin. About the larger vessels and bronchi the tissue was intensely edematous and infiltrated with wandering cells, including many polymorphonuclears. The lymphatic vessels were distended. In the unconsolidated portions of the lung the congestion was intense; there were hemorrhages and moderate alveolar edema. Pneumococci were not found at this stage.

The remaining three animals of this group, dying 2, 3, and $5\frac{1}{2}$ hours after injection, showed virtually the same lesions, save that there was extension of the pneumonic areas, and an increased amount of fibrin. At this time, however, pneumococci were already present in considerable numbers, and in one, which died after 2 hours, an amazing multiplication of the bacteria had occurred.

2. *Guinea Pigs Dying in from 18 to 72 Hours after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.*

These animals did not appear sick or appeared only slightly sick after the injection. On the following day, however, they were all extremely ill, suffering from severe dyspnea which became progressively worse until death. At autopsy, from $\frac{1}{5}$ to $\frac{2}{3}$ of the total volume of both lungs was found enlarged and consolidated. The lower lobes were most frequently affected but consolidation was often present also in the middle and upper lobes. In all but one case both lungs were involved; in this instance, however, the entire right lung was

consolidated, and the left normal throughout. The pneumonic areas were deep red, yellowish gray, or greenish gray, the gray portions adjoining the hilum and the red portions surrounding them. The normal lung tissue was always at the margins of the lobes. The cut surface of the gray areas was yellowish gray and granular. In several of the guinea pigs which died in approximately 2 days, there was a cellulitis of the chest or abdominal walls. Cultures of the heart and lungs of this group always showed profuse growth of pneumococci.

Histological Findings.—Thirty-one guinea pigs of this group were studied. Although there were slight individual variations as regards the extent of the lesions, the pathological changes were on the whole surprisingly uniform. In almost all instances the greater part of both lungs was in a state of pneumonic consolidation. As was evident from the gross appearance, whenever a portion of the lobe remained unconsolidated, it was that at the margins, or distal from the hilic region.

In the earlier stages, the picture was in all essentials identical with that in the corresponding stages of lobar pneumonia in man. The alveoli were filled with an exudate composed predominantly of polymorphonuclear leucocytes; large mononuclears were not abundant, and when present could not readily be distinguished with the methods used from the exfoliated epithelium. The alveolar cells still attached to the septal wall were greatly swollen, and their cytoplasm vacuolated. Fibrin was irregularly present, but on the whole less abundantly and conspicuously than in human pneumonia. The capillaries in the earlier stages were engorged with well preserved erythrocytes. With the progress of the lesion, and the passage of the red blood cells into the alveoli, they frequently appeared collapsed and empty. Their basement membrane often seemed swollen and thickened. In the later stages, the red cells—those within the capillaries as well as those forming part of the alveolar exudate—appeared to fuse into agglomerated masses staining intensely with eosin, and such fused masses of erythrocytes formed capillary thrombi and entered into the matrix of the alveolar exudate. In the latter stages of the lesions, the derivation of this pink-staining material in which were embedded the fragmented leucocytes, was not apparent, but by following successive phases, it seemed clear that the hemoglobin

masses from the fusion of red blood corpuscles were the source of this material. The usual picture of hemolysis, with the preservation of the shadows of the hemolyzed cells, was never seen; nor was the picture that of rhexis.

The leucocytes of the exudate in the earlier stages were swollen and hydropic—often with a distinct cell membrane and a watery clear cytoplasm. Later the nucleus underwent fragmentation. The fibrin, delicate at first, later became swollen and the threads merged into larger clumps and masses.

The most striking and interesting feature of these experimental lesions, however, was the unrestrained growth of the pneumococci. In all the animals of this group, enormous numbers of diplococci were present, in many alveoli forming dense colonial masses. In sections stained by the Gram-Weigert method, the bacteria were so numerous as to impart a blue color to the section. They were often present in abundance in the edematous tissue about the blood vessels and the bronchi, as well as in the alveolar exudate.

3. Guinea Pigs Which Survived 7 and 14 Days Respectively after the Intratracheal Injection of Pneumococcus Culture and Toxic Autolysates.

The guinea pig which died in 7 days was slightly dyspneic after the first day and continued in this condition until death. At autopsy, the lungs were collapsed but appeared otherwise normal except for the left lower lobe which was slightly consolidated and of a translucent grayish color. Cultures from the heart and left lower lobe of this guinea pig gave a profuse growth of pneumococci. Histologically, the section through the left lower lobe showed the capillaries to contain little blood; the endothelial nuclei were swollen and hyperchromatic, the alveolar epithelial cells large and vacuolated. In the connective tissue about the main bronchus were several circumscribed masses of polymorphonuclear cells—the only evidence of an acute inflammatory reaction. The edematous peribronchial tissue was becoming organized by the ingrowth of fibroblasts. The lesions indicated recovery from a previous pneumonia.

Guinea Pig 84 died on the 14th day, having shown no signs of illness at any time. No gross or microscopic lesions were found to indicate

the occurrence of a previous pneumonia. Either the animal was exceptionally resistant to the autolysate and pneumococci or there had been a technical error in the inoculation.

(C) Results from the Intratracheal Injection of Pneumococcus Cultures and Broth.

Ten guinea pigs were injected with mixtures of pneumococcus cultures and broth. None of these became dyspneic at any time; only three of them died, two on the 5th day and one on the 7th. At autopsy, the gross appearance of the lungs of these three was normal. Cultures from the heart blood yielded a profuse growth of pneumococci.

Histological Findings.—Ten guinea pigs were studied histologically, the three which died, described above, and seven additional guinea pigs which were killed at intervals of 1 to 4 days after the inoculation of varying amounts of culture. Lesions were found only in the animals which were killed during the first 48 hours. They consisted of a sparse emigration of polymorphonuclear leucocytes into a few alveoli only, accompanied by occasional red blood corpuscles. There was no fibrinous exudation. Indeed there was no massive consolidation comparable to that regularly occurring in the animals of Group B already described. Pneumococci were found in the sections of but two of this series—6-28, killed after 27 hours and 6-39, killed after 2 days—and not in excessive numbers. Both of these animals received a very much larger dose of the microorganism than was given to the animals of Group B, which received toxic autolysates in addition to the living pneumococci.

The three guinea pigs dying on the 5th and 7th days showed no pulmonary lesions whatsoever in spite of the general septicemia.

(D) Results from the Intratracheal Injections of Toxin and Broth.

Ten guinea pigs were injected with toxin and broth. Of these, three pigs died; two died 2 hours after the injection, and one died 48 hours after the injection with characteristic symptoms and pathology of a guinea pig injected with 0.2 cc. of a strong toxin, described above, *viz.*, in the two pigs which died in 2 hours the lungs were patchy deep red and pink throughout; in the pig which died in 48 hours $\frac{2}{3}$ of the

lungs was consolidated and deep red.   The cultures of the lungs and heart of these three pigs were sterile.   The other seven guinea pigs in this series were more or less dyspneic after the inoculation but had practically recovered from this sickness by the next day and survived.

<div align="center">DISCUSSION.</div>

If we contrast the symptoms and pathology in these three groups, it is evident that there are striking and significant differences in the reaction of the animal.   In agreement with previous workers it has been impossible to produce extensive pneumonic lesions by the injection of living pneumococci alone, even in large doses.   The lesions which followed the intratracheal administration of the living organism appeared early and were mild and transitory in character even when the doses were greatly in excess of those used in the other groups. The animals which succumbed died of septicemia without lung involvement.

In striking contrast to this behavior was the reaction of the animals which received in addition to the living pneumococci, a small dose of the toxic autolysate.   A certain proportion of these died within a few hours with shock-like symptoms; and from our observation upon the effect of toxic autolysate alone it would seem that the poisonous effect of this substance was primarily responsible since death occurred before there had been opportunity for the multiplication of the bacteria. The changes in the lung may be summarized as an intense hemorrhagic edema with very early inflammatory reaction.

In animals surviving this first shock there developed regularly massive areas of consolidation often of the lobar type and accompanied by an amazing multiplication of the pneumococci throughout the lung.   The growth of the bacteria seemed indeed to outstrip the inflammatory reaction, since one found them even in areas outside of the pneumonic zones.   It is clear that the toxic substances have in some way blocked the defensive mechanism and made conditions favorable for unrestrained growth.   We shall not at this time attempt to analyze this further, or speculate as to the factors responsible for this striking phenomenon.

Experiments also demonstrated the marked toxicity of the bacterial autolysates prepared after the described manner.   The lesions pro-

duced were intense and widespread in the lungs. The chief differences noted histologically between the reactions to the toxic autolysates alone, and the reaction when the poisonous substance was accompanied by the introduction of the living organism, are that there is less fibrin in the former group and the exudate appears to be somewhat looser in character. The alteration of the red blood corpuscles which was a striking feature in the presence of the living bacteria, was not seen with the toxic autolysate alone. Furthermore, as one might expect, there is an attempt at recovery and repair on the part of the animals which have been allowed to live for 3 days or more. These observations upon the primary toxicity of the autolysates brings up, of course, the interesting problem as to the rôle of similar substances formed *in vivo*, in the production of pneumonia in man.

CONCLUSIONS.

1. Anaerobic autolysates of pneumococci, prepared according to the method described, are highly toxic for guinea pigs when injected intratracheally in dosage of 0.2 cc. Death occurs either within a few hours (36 per cent) or within 3 days. In the early deaths there is intense hemorrhagic edema of the lungs with beginning inflammatory reaction; in animals surviving for 18 hours or longer extensive areas of pneumonia are produced.

2. The intratracheal injection of virulent living pneumococci is followed by transient slight lesion with recovery, or by later death from septicemia without pneumonic lesions.

3. The addition of a sublethal dose of toxic autolysate to living pneumococci alters the reaction of the animal, so that there develops extensive pneumonia associated with unrestrained multiplication of the organism.

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EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. G. P. 8-49.   Killed 29 hours after intratracheal injection of toxic autolysate alone.   Patchy areas of pneumonia and diffuse edema.

FIG. 2. G. P. 8-53.   Killed 24 hours after intratracheal injection of pneumococci alone.   Sparse emigration of polymorphonuclears into a few alveoli.   No pneumonic consolidation.

PLATE 19.

FIG. 3. G. P. 10-29.   Killed 48 hours after intratracheal injection of pneumococci plus toxic autolysate.   Diffuse pneumonic consolidation, with abundant growth of pneumococci.

FIG. 4. G. P. 6-27.   Died 24 hours after intratracheal injection of toxic autolysate and pneumococci.   Unrestrained growth of pneumococci in lung.   (Gram-Weigert stain.)

FIG. 1.
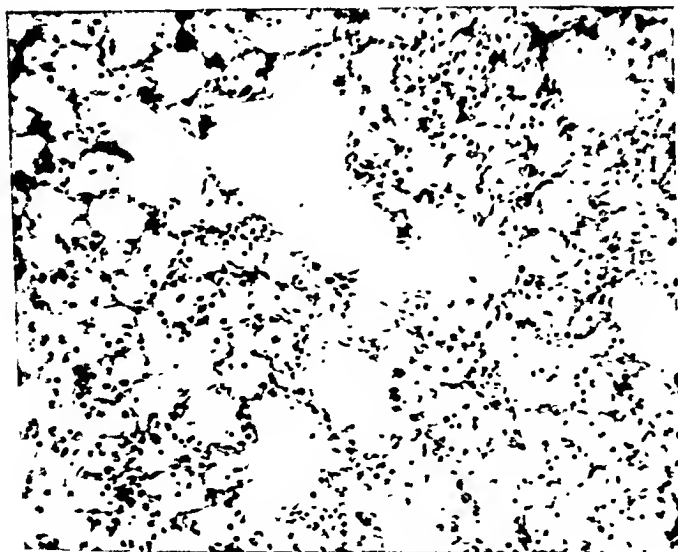
FIG. 2.

(Parker and Pappenheimer: Pneumonia in guinea pigs. L)

Fig. 3.

Fig. 4

STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

XI. An Inquiry into the Mode of Action of Antibacteriophage Serum.

By RALPH S. MUCKENFUSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

That sera of animals immunized by parenteral introduction of lytic filtrates have the property of neutralizing bacteriophage has been recognized since the work of Bordet and Ciuca in 1921 (1), but this demonstration of a new antigen was at the time of purely academic interest. In 1925, however, Sonnenschein (2), and shortly afterward Katsu (3), stated that human blood possessed antibacteriophagic properties in relation to certain infectious diseases, and they suggested that the reaction might be of diagnostic value. This possibility has made the serological study of bacteriophage of considerable practical importance.

Methods.

Purification of Phages.—It is a common observation that when a lytic filtrate is first obtained it ordinarily contains a number of different principles, or bacteriophages. The first procedure in the purification of this mixture is to carry lysis in series through a number of passages on the bacterial strain, thus eliminating by dilution any phages that act only on heterologous bacteria.

It is probable that even the filtrate thus obtained still consists of several different components which act on the same bacterial species, and which can be further separated by the plating method. For each phage forms a characteristic plaque, as has been demonstrated by Bail (4) and by Gratia (5). Plaques of the same sort should be picked for several passages, in this manner securing a pure phage.

Pure phages obtained in this manner were used as antigens. Immunization was conducted by intravenous and subcutaneous injections, and in both cases neutralizing sera were obtained. The sera of mice, on the other hand, that were fed phage daily for 6 weeks, were not capable of neutralizing the phage ingested.

Technique for Demonstrating Neutralization.—Bordet and Ciuca (1) demonstrated neutralization by adding a mixture of a specific serum and the appropriate phage to a culture of susceptible bacteria in broth, the absence of visible lysis serving as a criterion of neutralization. They also demonstrated (6) in a control experiment, that normal serum did not prevent phage action. D'Herelle and Eliava (7), using this technique, found that on prolonged incubation lysis took place, and so concluded that the serum merely inhibited the lysis, but that it did not inactivate the phage. Wagemans (8), however, found that if the time of contact between phage and serum was sufficiently long, neutralization was complete and the phage could not be regenerated. A technique commonly employed (9) has been to mix phage and serum for a varying length of time and then to streak this mixture on a plate seeded with susceptible bacteria, neutralization

TABLE I.

The Neutralization of Coliphage by Immune Serum.

Phage	Antigen of serum	Bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C.	Normal serum	*B. coli*	10^{-9}	+++	++	100±	—
	P.C. coliphage	" "	10^{-4}	—	—	—	—

+ + + indicates complete lysis.

+ +, +, indicate lesser degrees of lysis.

Numbers indicate the number of plaques.

± following a number indicates that the number of plaques was estimated.

— indicates absence of lysis.

These signs are used in all of the following protocols in which lysis on agar slants is recorded.

being demonstrable by the absence of plaque formation. Sonnenschein (10) did this in a quantitative manner, mixing diluted phage with undiluted serum on the one hand, and undiluted phage with diluted serum on the other, running drops of the mixtures down the previously inoculated surface of an agar plate and observing lysis after incubation.

In our search for a satisfactory technique the findings of Wagemans (8) were first repeated and confirmed. It was found, however, that the time required for complete neutralization depends on the potency of the serum, a serum of high potency causing complete neutralization in a comparatively short time. Inhibition of lysis in broth proved unsatisfactory as a method, since a partial neutralization can delay or prevent visible lysis, although increase of the phage may proceed at a fairly rapid rate. For detecting partial neutralization it has been found

TABLE II.

The Specificity of Antiphage Sera.

Phage	Antigen of serum	Bacterium	Undiluted Titer	Undiluted Slant	1-100 Slant	1-10,000 Slant	1-1,000,000 Slant
P.C.	Normal	B. coli	10^{-9}	+++	++	+	14
	P.C. phage		10^{-5}	−	−	−	−
	Laudman phage		10^{-9}	+++	++	+	11
	P.I D. "		10^{-10}	+++	++	+	5
	B.H. "		10^{-10}	+++	++	+	8
	Normal	B. dysenteriæ Shiga	10^{-9}	+++	+++	+	15
	P.C. phage		10^{-6}	−	−	−	−
	Laudman phage		10^{-9}	+++	+++	+	20
	P.I.D. "		10^{-9}	+++	+++	+	25
	B.H. "		10^{-9}	+++	++	+	6
Laudman	Normal	B. coli	10^{-10}	+++	++	+	3
	P.C. phage		10^{-9}	+++	++	+	4
	Laudman phage		10^{-3}	−	−	−	−
	P.I.D. "		10^{-10}	+++	++	+	−
	B.H. "		10^{-9}	+++	++	+	3
	Normal	B. dysenteriæ Shiga	10^{-9}	+++	+++	++	16
	P.C. phage		10^{-10}	+++	+++	++	7
	Laudman phage		10^{-5}	−	−	−	−
	P.I.D. "		10^{-10}	+++	+++	++	8
	B.H. "		10^{-9}	+++	+++	++	−
P.I.D.	Normal	M.T.²	10^{-8}	+++	++	+	12
	P.C. phage		10^{-8}	+++	++	+	−
	Laudman phage		10^{-8}	+++	++	+	7
	P.I.D. "		10^{-2}	−	−	−	−
	B.H. "		10^{-7}	+++	++	+	10
B.H.	Normal		10^{-8}	+++	++	100±	1
	P.C. phage		10^{-8}	+++	++	100±	−
	Laudman phage		10^{-9}	+++	++	100±	−
	P.I.D. "		10^{-8}	+++	++	100±	3
	B.H. "		10^{-2}	−	−	−	−

convenient to drop a mixture of phage and antiserum on an evenly inoculated agar surface, and to compare the resulting lysis with that caused by a mixture of phage and normal serum. Absence of lysis under these conditions, however, does not necessarily indicate complete inactivation of the phage.

As the reaction of neutralization may at times continue for several days, it was found best to limit the time of contact of phage and serum to a fixed period and then to determine quantitatively, by the titration method of Appelmans (11), the amount of phage remaining active. In addition to this, serum was mixed with serial dilutions of the phage, and after the given period of contact a drop of each

TABLE III.

The Specificity of Neutralization of Adapted Phages.

Phage	Antigen of serum	Susceptible bacterium	Phage dilution				
			Undiluted		1–100	1–10,000	1–1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. phage adapted to Shiga bacillus	Normal	B. coli	10^{-8}	+++	++	+	2
	P.C. phage		10^{-3}	−	−	−	−
	Laudman phage		10^{-8}	+++	++	+	11
	Normal	B. dysenteriæ Shiga	10^{-8}	+++	++	100±	2
	P.C. phage		10^{-3}	−	−	−	−
	Laudman phage		10^{-8}	+++	++	100±	1
Laudman phage adapted to B. coli	Normal	B. coli	10^{-10}	+++	+++	150±	5
	P.C. phage		10^{-9}	+++	+++	150±	5
	Laudman phage		10^{-3}	−	−	−	−
	Normal	B. dysenteriæ Shiga	10^{-10}	+++	+++	+	14
	P.C. phage		10^{-10}	+++	+++	+	20
	Laudman phage		10^{-4}	−	−	−	−

of these mixtures was run down the surface of agar slants previously inoculated with the susceptible bacterium. The results with immune serum were then compared with those with normal serum. A typical protocol is given in Table I, which shows the neutralization of P.C. coliphage by its specific antiserum.

EXPERIMENTAL.

Specificity of Neutralization.—Maisin (12), in 1921, stated that antilytic sera are not specific in their action, but this finding lacks confirmation. In most of the experiments here to be reported four bacteriophages were used. They are listed below and their respective activity is indicated.

1. P.C. coliphage, acting on *B. coli* and *B. dysenteriæ* Shiga.

2. Laudman Shiga phage, acting on *B. coli* and *B. dysenteriæ* Shiga.

3. P.I.D. M.T.[2] phage, acting on M.T.[2] (*B. pestis caviæ*).

4. B.H. staphylococcus phage, acting on *Staph. aureus* "G."

In addition to these, B.W. coliphage, acting on *B. coli*, was used in some experiments. Neutralizing sera were prepared for each of the phages, and then the ability of each antiserum to neutralize each of the phages was determined. The result of these studies is given in Table

TABLE IV.

Specificity of Antisera for Different Phages Acting on the Same Bacterium.

Phage	Antigen of serum	Bacterium	Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
P.C. coliphage	Normal	B. coli	10^{-9}	+++	++	+	14
	P.C. phage	" "	10^{-5}	−	−	−	−
	Laudman phage	" "	10^{-9}	+++	++	+	11
	B.W. "	" "	10^{-9}	+++	++	+	12
Laudman coliphage	Normal	" "	10^{-10}	+++	+++	150±	5
	P.C. phage	" "	10^{-9}	+++	+++	150±	5
	Laudman phage	" "	10^{-4}	−	−	−	−
	B.W. "	" "	10^{-9}	+++	+++	150±	10
B.W. coliphage	Normal	" "	10^{-7}	+++	++	4	−
	P.C. phage	" "	10^{-7}	+++	++	2	−
	Laudman phage	" "	10^{-6}	+++	++	3	1
	B.W. "	" "	10^{-4}	1	−	−	−

II, in which it is seen that each serum exerts a specific neutralizing action on its own antigen, though it is without influence on the other phages.

The specificity of neutralization after the phage had been adapted to another bacterium was next studied. Seiffert (13) found neutralization to be specific after adaptation. Wagemans (8), Wolff and Jansen (14), and Kasarnowsky and Tiomkin-Schukoff (15), on the other hand, found that while specific neutralization usually occurred, this was not invariably the case. The results of our inquiry into this

TABLE V.

The Effect of Antibacterial Sera on Phage.

Phage	Antigen of serum	Bacterium	Undiluted Titer	Undiluted Slant	1-100 Slant	1-10,000 Slant	1-1,000,000 Slant
P.C. coliphage	Normal	*B. coli*	10^{-10}	+++	++	+	6
	B. coli	"	10^{-9}	+++	++	+	1
	" autolysate	"	10^{-9}	+++	++	+	8
Laudman Shiga phage	Normal	" *dysenteriæ* Shiga	10^{-9}	+++	+++	++	12
	B. dysenteriæ Shiga	" "	10^{-9}	+++	+++	++	10
	" autolysate	" "	10^{-9}	++	++	++	6
P.I.D. M.T.² phage	Normal	M.T.²	10^{-7}	++	+	20	—
	M.T.²	"	10^{-7}	++	+	20	—
	" autolysate	"	10^{-7}	++	+	15	—
B.H. staphylococcus phage	Normal	*Staph. aureus* G	10^{-7}	+++	++	100±	—
	Staphylococcus G	" "	10^{-6}	+++	+++	100±	—
	" autolysate	" "	10^{-6}	+++	+++	100±	—

question are given in Table III, and show that, with the material at our disposal, neutralization was specific for the original phage, even though it may have been carried for a number of passages on another bacterial species. The use of imperfectly purified phages may explain some of the discrepancies in the findings quoted, but the possibility that the antigenic properties of a phage may alter with adaptation to another bacterial species cannot be excluded as a property of some phages.

The specificity of the neutralizing power of sera prepared against different phages acting on the same bacterium was also studied. Bruynoghe and Appelmans (16), and Bail and Watanabe (17) described two serologically distinct phages acting on the same bacterial

TABLE VI.

Agglutination of Bacteria by Antiphage Sera.

Serum	Bacterium	1-10	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	1-5120	1-10,240	Control
Anti P.C. coliphage	B. coli	—	—	—	—	—	—	—	—	—	—	—	—
" Laudman Shiga phage	B. dysenteriæ Shiga	—	—	—	—	—	—	—	—	—	—	—	—
Anti P.I.D. M.T.² phage	M.T.²	+++	+++	+++	++	++	+	+	+	+	±	—	—
Anti B.H. staphylococcus phage	Staphylococcus	+++	+++	+	+	+	±	—	—	—	—	—	—

species. A later observation of interest is that of Watanabe (9) who found two phages, acting on the same bacterial species, that were similar serologically but distinguishable by other means. In our experiments P.C., Laudman, and B.W. phages, all acting on *B. coli*, were used, and the serum for each was found to be specific for its own antigen and without effect on the other two phages (Table IV).

The Effect of Antibacterial Sera on Phage.—Bail (18), in 1921, found that antibacterial sera neutralized phage, but that they were only one hundredth as strong as homologous antilytic sera. This finding has not been confirmed in other laboratories. That antibacterial sera in sufficient concentration can inhibit lysis in broth has been recognized (19), but this has been shown (20) to be due to a direct

action of the serum on the bacteria, and not to a process of neutralization of phage.

We have investigated the question thus brought up, and have used not only agglutinating sera, but also precipitating sera prepared against *autolyzed broth cultures of bacteria*. The results are given in Table V, which shows that no neutralizing antibodies were present in the sera.

However, when lysogenic cultures were used as antigens the antisera obtained exhibited definite antilytic properties. This experiment was carried out as follows:

Bacteria were made artificially lysogenic by continuing incubation after lysis in broth, and inoculating the overgrowth of resistants directly onto agar slants. When animals were immunized with these cultures, their serum, in addition to its agglutinating properties, became capable of neutralizing the phage carried; but here, of course, phage was also present in the antigen.

Presence of Other Antibodies in Antiphage Sera.—Since the filtrates used as antigens contained, in addition to the active bacteriophage, bacterial protein liberated during growth and subsequent lysis, the appearance of antibacterial antibodies was to be expected. These appeared with regularity, and antilytic sera prepared by immunization of animals with crude filtrates of lysed cultures regularly formed a precipitate when mixed with autolysates of the homologous bacterium, or with the filtrate used as antigen. Bruynoghe and Dubois (21) have found that precipitation is to some extent specific for the bacteriophage. In our hands the method has been unsatisfactory, since with the presence of degradation products of bacteria of closely related species there is enough non-specific precipitation to obscure the results.

The presence of agglutinins in antilytic sera is usual (22, 23). Marshall (24), however, prepared a serum that did not agglutinate the homologous bacterium. The results of our studies have been variable. From Table VI it will be seen that sera against the coliphage and the Shiga phage did not agglutinate the homologous bacteria, while those against M.T.[2] phage and staphylococcus phage were definitely agglutinating. In the case of staphylococcus agglutination occurred with normal sera also, so that the results are not so striking as the truly specific reactions obtained with M.T.[2]

Complement fixation was studied by Sanderson (25) and later by Flu (26). Their findings showed that with rabbit serum complement is fixed non-specifically in the presence of bacterial protein and the reaction is not related to the presence of bacteriophage; occasionally complement may be fixed even with plain broth. In view of these reports complement fixation was not tested by us.

Independence of the Two Types of Antibodies.—That the antibodies against the bacteria and those against the phage are distinct can be readily demonstrated by the absorption test. The antibodies against the bacteria may be absorbed, leaving unreduced the antilytic power of the serum. This fact has been observed in several laboratories (23, 27). It is also possible to remove a precipitate resulting from a mixture of a bacterial autolysate and antilytic serum without reducing the neutralization titer of the serum.

The Reaction of Neutralization.—In order to get some conception of the nature of the reaction when bacteriophage is neutralized by antiserum, a study of the factors entering into the reaction was made.

(a) *Rate of Neutralization.*—When phage and antiserum are placed in contact with each other, neutralization proceeds at first rather rapidly, but the rate of this process gradually decreases. Seisser (28) found that the greater part of the phage is neutralized within 24 hours, and Wagemans (8) found that complete neutralization may require as long as 4 days. Higher temperatures, according to Otto and Munter (29), increase the rate of neutralization. This was confirmed by Arnold and Weiss (30).

All of these findings have been confirmed in our laboratory.

Arnold and Weiss found that neutralization follows the law of multiple proportions and also reported the Danysz-Bordet phenomenon (30).

(b) *The Rôle of Complement.*—Osumi (31) found complement necessary for neutralization of bacteriophage by immune serum, but this finding has not been confirmed by other workers, and in our experiments complement played no part in the reaction.

(c) *Mode of Neutralization.*—Seiffert (13) reported that neutralization proceeds by a reduction in size, rather than number, of the plaques. Grigorieff (32), on the contrary, found that serum acts by inactivating individual units. With the material used in the present

work the latter has been found to be the mode of action. Reduction in size of the plaques has not been observed.

(d) *Nature of the Reacting Substances.*—That the action of the serum is directly on the bacteriophage and not on the bacteria or bacterial products has been demonstrated in the preceding pages by the specificity of their neutralization, by the failure of antibacterial sera to affect them, and by the undiminished antilytic activity of neutralizing sera after the complete removal of antibacterial antibodies.

(e) *The Influence of Adsorption.*—There is a possibility that phage may be inactivated by adsorption onto a precipitate which is formed when antiserum is combined with a filtrate containing bacterial protein. This possibility was tested by means of attempts to remove phage from a filtrate by the formation of a heterologous precipitate. Horse serum and homologous immune rabbit serum were mixed in the presence of phage and incubated overnight. The tube was then centrifuged and the clear supernatant fluid titrated. The lytic activity was found equal to that of another sample of phage similarly diluted with normal serum. Moreover, precipitation of filtrates by antibacterial sera failed to reduce the titer of phage. For these reasons it seems unlikely that adsorption can be the explanation of neutralization.

(f) *Reversibility of the Neutralization.*—The view that the neutralization of phage is analogous to the neutralization of toxin by antitoxin has been offered by certain workers who present evidence that the combination can dissociate. Otto and Munter (29) reported that dilution dissociated the combination but their method does not exclude the possibility of incomplete neutralization as the explanation. Weiss (33) has reported the complete dissociation of a neutral mixture by tryptic digestion.

We have attempted, though without success, to effect dissociation (1) by changing the reaction, and (2) by digestion with trypsin.

1. Dissociation by Changing the Reaction.—After determining the activity of a serum it was mixed with phage in the proportion to secure a neutral mixture (in this case 1–10). The resulting inactive fluid was diluted 1–5 in buffers of various degrees of acidity or alkalinity and these mixtures were left at room temperature overnight, when they were tested for phage. Phage alone, and serum alone similarly diluted and treated in an identical manner, were found to be unaffected. Table VII shows that dissociation was not secured by this method.

2. *Dissociation by Digestion with Trypsin.*—Trypsin solution was prepared by suspending 2 per cent of Fairchild's trypsin in borax-boric acid buffer at pH 7.4, and placing the flask in the ice box overnight. This material was then filtered through a Berkefeld V candle, and the reaction adjusted colorimetrically with

TABLE VII.

Attempt to Dissociate a Neutral Phage-Antiphage Mixture by Changing the Reaction.

pH of buffer.........	4.05	4.99	5.94	6.43	7.03	7.54	7.96	8.97	9.90
Quantity of buffer, cc.................	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Quantity of neutral mixture, cc........	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Mixtures left at room temperature overnight									
Titer...............	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

TABLE VIII.

Effect of Trypsin on Neutral Phage-Antiphage Mixture.

Day of incubation.........................	Immediate	1	2	4	5	6
Phage with normal serum...........	10^{-3}	10^{-5}	10^{-5}	10^{-6}	10^{-7}	10^{-5}
" " immune "	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

TABLE IX.

Effect of Trypsin on Antiphage Serum.

Serum	Undiluted		1-100	1-10,000	1-1,000,000
	Titer	Slant	Slant	Slant	Slant
Normal............................	10^{-9}	+++	++	150±	4
Immune before digestion..............	Neg.	—	—	—	—
Digested 1 day.......................	10^{-6}	+	25±	—	—
" 2 days......................	10^{-7}	++	100±	1	—
" 4 "	10^{-7}	++	150±	1	—
" 6 "	10^{-7}	++	100±	—	—

sterile sodium hydroxide to a pH of 7.4. One volume of this solution was added to three volumes of a neutral mixture of coliphage with the corresponding anti-serum. Phage with normal serum was similarly treated as a control, and the two tubes were placed in the incubator at 37°C. The solution of trypsin was shown

to be active in a duplicate tube to which a Mette tube was added.   The activity
of the phage in each of the two tubes was determined at intervals (Table VIII).
At the same time, immune serum was similarly digested with trypsin and its
ability afterwards to neutralize phage was determined at the same intervals
(Table IX).

The neutralizing power of the serum alone was markedly diminished by the
trypsin, but, in spite of this, the phage was not liberated from a neutral mixture
with this same serum by the action of trypsin.   These experiments were repeated
with other phages and antisera, but no definite evidence of dissociation was
obtained.

That neutral mixtures may dissociate under certain conditions is suggested,
however, by the fact that when rabbits are immunized with a neutral mixture of
phage and immune rabbit serum, their serum becomes capable of neutralizing
phage.

Stability of the Bacteriophage Antigen.—Arnold and Weiss (34), and
Asheshov (35) have shown that phage inactivated by heat is still
antigenic. We have confirmed this. However, boiling, or autoclaving
at 120°C. for 1 hour renders the phage antigenically inert, as deter-
mined by the usual course of immunization. After inactivation of
phage by formalin, by immune serum, or by adsorption onto dead
bacteria, its antigenic property remains unchanged.

The Occurrence of Neutralizing Antibodies in Disease.—As stated
previously, Sonnenschein (2) and Katsu (3) detected antilytic proper-
ties of human sera and related these findings to specific infections.
The subject has been studied experimentally in this laboratory by
feeding the bacteria of mouse typhoid (M.T.[2]) to mice and testing the
serum of those animals surviving the infection for neutralizing anti-
bodies. Three phages acting on this bacterium were available, and
none of these was neutralized by the serum of the surviving mice.
Moreover, the spontaneous appearance of neutralizing antibodies in
experimental animals has never been observed in this laboratory.

DISCUSSION.

The reaction between bacteriophage and specific neutralizing anti-
serum constitutes a definite serological phenomenon, different from and
independent of any of the known reactions between bacteria or bac-
terial products and their antisera. Each bacteriophage is a specific
antigen independent from the bacterial antigens present in lytic

filtrates, and the antigenic properties are affected by the same influences that affect other known antigens, as for example heat. The reaction of neutralization seems to be analogous to the neutralization of toxin by antitoxin, in that complement is unnecessary, neutralization is not instantaneous but takes some time, and the reaction follows the law of multiple proportions. The fact that each phage is neutralized only by its specific antiserum makes possible the serological identification and classification of bacteriophages.

Antilytic sera may at times be of some value in the laboratory. A mixture of phages may be partially purified by using a serum which neutralizes one or more components, leaving the remainder active and thus simplifying the problem of their separation. Lysogenic cultures may be rendered free of phage by cultivation in broth containing serum active against the phage carried. The ensuing neutralization of phage permits the culture to return to its original state.

The use of the reaction of neutralization in the diagnosis of disease seems, in view of the results reported here, to be impractical. The failure of antibacterial sera to neutralize phage, and the indefinite number of phages acting on the same bacterial species would render the test uncertain. Furthermore, only positive results would be of any significance, since it would be difficult to exclude the presence of antibodies against some other bacteriophage acting on the same bacterium.

SUMMARY.

1. Each bacteriophage is a specific antigen.

2. The antibodies against the bacteriophage are independent of those against the bacterial substrate used in preparing the phage.

3. The reaction of neutralization is closely analogous to that of the neutralization of toxin by antitoxin.

4. The serum of mice experimentally infected with mouse typhoid (M.T.[2]) did not become capable of neutralizing phages acting on the infecting organism.

5. The use of the reaction of neutralization of bacteriophage in the diagnosis of disease as proposed by Sonnenschein seems impractical.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

XII. Concerning the Production of Phage from Bacterial Cultures.

By RALPH S. MUCKENFUSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The development of bacteriophage in bacterial cultures has been described repeatedly. The importance of the phenomenon is self-evident. If the production of phage from bacteria themselves can be incontrovertibly proved, then it is scarcely conceivable that the bacteriophage can be a living ultramicroscopic parasite of the bacteria.

In some cases, plaques from which lysis in series can be conducted have appeared spontaneously in stock cultures (1). In most cases, however, a more complicated procedure has been necessary (2) for the development of the phage, one involving repeated filtrations and reinoculations, sometimes of young cultures, but usually of cultures that have aged or been treated with some chemical or physical agent. The objection to all of the results is that they cannot be reproduced at will, and although positive results may predominate in the hands of some workers, the outcome of individual experiments is uncertain. Furthermore, the amount of manipulation necessary increases the possibility of contamination with phage, and this factor becomes of increasing importance when one considers the ubiquity of phage and its relative resistance to most chemical and physical agents. Consistent results were reported at one time by Putter and Vallen (3), but they subsequently traced them to the use of contaminated filters (4), and therefore retracted their previous statements. The possibility that bacteriophage was already present in the cultures used must also be considered. In this connection Manninger (5) takes the extreme view that all of the cultures of the colon-typhoid-paratyphoid group of bacteria are contaminated with bacteriophage.

A phenomenon distinct from these, however, and one which can be

regularly brought about is the production of phage for one bacterium in cultures of another.

This was first described in 1922 by Lisbonne and Carrère (6), and they considered it the result of bacterial antagonism. Kuttner (7), in 1923, produced phage from stock cultures, and the phage produced acted only on heterologous strains, and not on the strain from which it was derived. In 1924, Gildemeister and Herzberg (8) described a bacterium (*Coli* 88) having the property, like the *B. coli* strain of Lisbonne and Carrère, of initiating serial lysis of the Shiga bacillus. These two bacterial strains have similar, if not identical, properties, so the literature on the two will be summarized together.

Bordet was able, by culturing from single colonies, to secure strains that would not produce phage for the Shiga bacillus (9), and by subculturing single colonies for a number of passages, he could get strains that were to some extent lysogenic for each other (10). Since the great majority of the strains from single colonies could still produce phage for the Shiga bacillus, he called this phenomenon "active lysogenesis," as contrasted to "passive lysogenesis" which resulted from artificially mixing a culture with phage, and in which the phage was only perpetuated in mass cultures.

D'Herelle (11) repeated Bordet's experiments, and found that in artificially produced lysogenic cultures phage persisted in transfers from single colonies if the "rough" colonies were selected. He thought that such colonies indicated symbiosis of the bacteriophage with the bacteria. Bail (12) confirmed d'Herelle's experiments and agreed with him that the phenomenon did not represent a true production of phage from the bacteria themselves but resulted from the admixture of phage with the bacteria. McKinley (13) immunized animals with broth cultures of the *coli* strain of Lisbonne and Carrére and the resulting sera neutralized the phage already produced by the culture, but would not render the strain of bacteria non-lysogenic.

To explain the phenomenon of phage production for the Shiga bacillus in cultures of *B. coli* on the basis of phage preexisting in the culture, it is necessary to assume that the colon bacillus employed for the work carries a phage active against both *B. coli* and against *B. dysenteriæ* Shiga, and that the particular strain of *B. coli* employed is resistant to this phage to such an extent that no gross lysis occurs, although enough individuals in the culture prove susceptible to serve for the perpetuation of the phage. It should be possible to reproduce the phenomenon experimentally, and this we have attempted to do in the following work. In addition to using a phage acting on both *B. coli* and *B. dysenteriæ* Shiga, a monovalent phage, acting only on *B. coli*, was used. In the case of this latter the original strain of bacteria was necessary to demonstrate the presence of phage.

EXPERIMENTAL.

The Production of Lysogenic Bacteria.—B. coli, and P.C. phage, which acts both on *B. coli* and on *B. dysenteriæ* Shiga, were inoculated into a tube of broth, and incubation was continued after the completion of lysis. The resulting overgrowth was inoculated directly onto agar slants. The growth appeared normal, but phage was readily demonstrated by cultivating in broth either with *B. dysenteriæ* Shiga or with the original strain of *B. coli*, then removing the living resistant bacteria by filtration, centrifugation, or heating to 56°C. for 40 minutes, and adding some

TABLE I.

Serological Identification of Phage Recovered from Lysogenic Bacteria.

Phage derived from:	Serum	Bacterium	Phage dilution			
			Undiluted	1–100	1–10,000	1–1,000,000
P.C. lysogenic *B. coli*	Normal	*B. coli*	+++	++	100±	–
	Anti P.C. phage	" "	–	–	–	–
	" B.W. "	" "	+++	++	100±	–
B.W. lysogenic *B. coli*	Normal	" "	+++	+++	26	1
	Anti P.C. phage	" "	+++	+++	23	1
	" B.W. "	" "	++	++	–	–

+++ = complete lysis.

++, + = lesser degrees of lysis.

Numbers indicate the number of plaques.

± following a number indicates that the number was estimated.

– indicates absence of lysis.

These signs will be used in the following protocols whenever lysis on agar slants is recorded.

of this material to a fresh culture of the susceptible organism. Phage could in this way be demonstrated even after the bacteria had been cultivated in series through 80 daily passages. A like experiment was done with B.W. coliphage, which acts only on *B. coli*. The phage could be demonstrated by allowing the resulting lysogenic strain to act on the original strain of colon bacilli.

Identification of the Phages Produced by Lysogenic Bacteria.—It was necessary to ascertain that the phages produced were those to which the bacteria were originally exposed. This was readily done, as the two phages are distinct serologically, and a serum prepared against one of the phages is inactive against the other.

Serial dilutions of the phages recovered were mixed with equal quantities of sera prepared against the original phages, and after an interval of 1 hour at room temperature, drops of the mixtures were run down the center of agar slants previously inoculated with the susceptible organism (Table I).

The phage recovered from the culture made lysogenic by exposure to P.C. phage was found to be neutralized by anti P.C. phage serum and not by anti B.W. phage serum, while the reverse was true of the phage recovered from the culture exposed to the action of B.W. phage. This establishes the identity of the phages recovered from the bacteria with those to which the cultures were originally exposed.

Serological Detection of Phage in Lysogenic Cultures.—It seemed reasonable to

TABLE II.

Phage Neutralization by Sera Prepared against Lysogenic Bacteria.

Phage	Serum	Bacterium	Phage dilution			
			Undiluted	1-100	1-10,000	1-1,000,000
P.C. coliphage	Normal	*B. coli*	+++	++	—	—
	Anti killed P.C. lysogenic	" "	—	—	—	—
	" live " "	" "	—	—	—	—
B.W. coliphage	Normal	" "	+++	++	60±	—
	Anti killed B.W. lysogenic	" "	++	40±	1	—
	Anti live B.W. lysogenic	" "	—	—	—	—

assume that if phage is present in a culture, it should act as an antigen, and consequently, an animal immunized against a lysogenic culture should give a serum capable of neutralizing the phage carried. Animals were therefore immunized by intravenous injection, with the following antigens:

1. P.C. lysogenic *B. coli*, 18 hour broth culture.
2. P.C. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.
3. B.W. lysogenic *B. coli*, 18 hour broth culture.
4. B.W. lysogenic *B. coli*, heat-killed saline suspension from an 18 hour agar slant culture.

The sera of the immunized animals were then tested for ability to neutralize P.C. and B.W. coliphages, and the results are recorded in Table II. From this it is seen that the sera of animals immunized with lysogenic cultures are capable

of neutralizing the phage carried. It has been shown in a previous communication (14) that antibacterial sera are without effect on phage, so the ability of an antibacterial serum to neutralize bacteriophage must indicate that the bacteriophage was present in the culture used as antigen.

An attempt was then made to carry out analogous experiments with a culture of colon bacilli that was lysogenic when isolated. A culture of the *B. coli* strain of Lisbonne and Carrère was kindly furnished us by Dr. E. B. McKinley, and a rabbit was immunized by intravenous injections of 18 hour broth cultures, freshly prepared for each injection. After the course of immunization usually followed in this laboratory (3 daily injections at weekly intervals for 4 weeks) the serum was tested for ability to neutralize the phage secured by the action of this strain of bacteria on the Shiga bacillus.

TABLE III.

Neutralization of Phage by Serum against B. coli Strain of Lisbonne and Carrère.

Phage	Serum	Bacterium	Phage dilution				
			Undiluted		1-100	1-10,000	1-1,000,000
			Titer	Slant	Slant	Slant	Slant
Phage from *coli* strain of Lisbonne and Carrère	Normal Anti *B. coli* of Lisbonne and Carrère	Shiga L " "	10⁻⁸ 10⁻⁶	++ +	+ 30±	4 —	— —

Table III shows that, while the degree of neutralization was not great, there was definite neutralization of the phage by the immune serum.

This is considered definite evidence that the phage was present in the culture. The antilytic property of serum of animals immunized against this bacterium has also been reported by McKinley (13) and confirmed by da Costa Cruz (15).

The phenomenon, as experimentally reproduced, consists in the demonstration of the phage which was previously mixed with the bacterial culture, and which remains present through an indefinite number of transfers.

DISCUSSION.

Consistent production of phage by one bacterium for another seems, in view of the experiments recorded here, to be the result of contamination of the "active" strain with a bacteriophage capable of acting on the susceptible strain.   The majority of the individuals in the lysogenic strain are resistant and therefore such a culture cannot be used in the demonstration of the phage carried, unless, by picking a sufficiently large number of isolated colonies, a susceptible strain can be obtained. This has been done by Bordet, though his explanation does not agree with the one here given.

The phenomenon is readily reproducible experimentally by exposing a bacterial culture to the action of a polyvalent phage, the lysogenic strain thus obtained producing a phage that has the same range of activity as that possessed by the original phage.   The apparent production of phage consists merely in the demonstration of the presence of the phage previously added, and that persists in the culture indefinitely.   To demonstrate more conclusively that the phage recovered is the same one originally added to the culture, advantage can be taken of the fact that each phage is a specific antigen, and so the phage recovered can be identified with the original phage by its neutralization by specific antiserum.

Furthermore, the presence of phage in the bacterial culture may be detected by the immunization of animals, the resulting antibacterial serum being also capable of neutralizing the phage carried.   As antibacterial sera are known to be incapable of neutralizing bacteriophage, the presence of neutralizing antibodies may be considered evidence that phage was present in the culture.   The absence of neutralizing antibodies in an antibacterial serum, however, cannot be taken as positive evidence of the absence of bacteriophage, as antigens vary in their ability to stimulate antibody production.   The possibility must be considered, therefore, that the phage carried may be a poor antigen and that no detectable antilytic properties may develop during the course of immunization.   The development of antilytic properties during the immunization of animals with the $B.$ $coli$ strain of Lisbonne and Carrère shows definitely that phage was present in the culture.

The production of phage from bacterial cultures alone must be regarded as unproven, though this possibility must still be considered.

SUMMARY.

1. The phenomenon of phage production by one bacterial culture for another of different sort has been reproduced experimentally.

2. This phenomenon results from phage carried with the culture, and not from the spontaneous appearance of phage in a culture previously free from it.

3. Animals immunized against the lysogenic bacteria may develop antibodies that neutralize the phage carried.

4. The development of neutralizing antibodies on immunization with a bacterial culture is evidence of the presence of bacteriophage in the culture.

5. The failure of such antibodies to appear on immunization with bacteria does not necessarily indicate that bacteriophage is not present.

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ON THE INHERITANCE OF AGGLUTINOGENS OF HUMAN BLOOD DEMONSTRABLE BY IMMUNE AGGLUTININS.[1]

By K. LANDSTEINER, M.D., and PHILIP LEVINE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Studies on the inheritance of serological properties were first undertaken systematically by von Dungern and Hirschfeld with the agglutinable substances in the blood of dogs (2) and with the human isoagglutinogens (3).[2] The authors named discovered the fact that the isoagglutinogens A and B are inherited as Mendelian dominants and this result has been amply confirmed by numerous workers.

According to their hypothesis there are two pairs of allelomorphic genes, *Aa*, and *Bb*, where *A* and *B*, the dominant genes, determine the presence of the corresponding agglutinogens, and *a* and *b*, the recessive genes, their absence. The genes for the blood groups are the following; group O:[3] aabb; group A: AAbb or Aabb; group B: aaBB or aaBb; group AB: AABB or AABb or AaBB or AaBb.

Another hypothesis has been advanced by Bernstein (6). He assumes multiple (three) allelomorphs, R, A, and B. The genetic formulæ accordingly are; group O: RR; group A: AA, AR; group B: BB, BR; group AB: AB. The theory of Bernstein does not involve a deviation from the older theory in the types of offspring except in the cases of parents belonging to group AB. According to the older view there may be children of any group in unions where one or both of the parents are in group AB; Bernstein's hypothesis, on the other hand, excludes children of groups O and AB in unions O × AB, and children O in unions A × AB, B × AB, or AB × AB. The recent work especially of Schiff (7), Thomsen (8), Preger (9), and Sievers (10) supports the opinion of Bernstein.[4,5]

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[1] See the preliminary report (1).

[2] The problem of the inheritance of the human blood groups and a few results had been mentioned by Ottenberg and Epstein (4).

[3] The nomenclature of the blood groups by letters instead of numerals has been recommended both by the American Association of Immunologists and by the National Research Council (5) and is used in the present publication.

[4] The objection of Mendes-Correa (11) to the theory of Bernstein would imply

Tests for M and N in Several ·

Family No.	143						144				
Blood No.	F 298	M 299	300	301	302	303	F 304	M 305	306	307	308
Group	A	A	A	A	A	A	O	O	O	O	O
Reaction for M	+++	+++	++±	++±	+++	+++	++±	++±	++±	++±	+++
Reaction for N	−	−	−	−	−	−	+±	+±	+±	+±	−

The strength of the reactions is indicated by the signs +, +±, ++, ++±, +++.

TABLE II.

Heredity of the Agglutinogen M.

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		M+	M−	M+	M−
M+ × M+	101	403	33	92.4	7.6
M+ × M−	59	165	85	66.0	34.0
M− × M−	6	0	29	0	100

E I.

lies. *F = Father; M = Mother.*

	145						146						147				
F 310	M 311	312	313	314	315	F 316	M 317	318	319	320	321	F 322	M 323	324	325	326	327
O	A	A	A	A	A	O	A	O	A	A	O	A	O	A	A	O	A
–	++	++	+++±	–	++±	++	+++±	+++±	+++±	+++±	+++±	–	+++±	–	+++±	–	–
++	+±	÷	+	++	+±	–	++	+±	+±	–	–	+++±	++	+++±	+±	+++±	++

TABLE III.

Heredity of the Agglutinogen N.

Type of parents	No. of families	No. of children of type		Per cent of children of type	
		N+	N–	N+	N–
N+ × N+	31	130	18	88.5	11.5
N+ × N–	29	81	40	66.9	33.1
N– × N–	4	0	17	0	100

The investigations outlined on the heredity of human blood groups are not only of theoretical interest[5] but they have attracted much attention because of their practical application in forensic medicine. A certain limitation lies in the fact that only two properties could be utilized. It is true that some experiments pointed to the existence of differences in human blood aside from the blood groups (13–16), but as a result of these studies no genetic investigations worthy of notice have been reported although obviously such would have been desirable. The reason for this is to be seen in the lack of workable methods.

The observations reported in previous publications (1, 17, 18) enabled us to undertake a study of the heredity of serological properties of human blood other than those determining the four blood groups.

With regard to the property designated as M there was no difficulty in selecting immune sera and absorbing bloods in such a manner that the reactions were either entirely negative on microscopic examination or so strong that clumps were visible to the naked eye.

The results with a second property, N, whose heredity was studied, varied depending upon the particular immune serum used. Although the strongest agglutinations occurred with the same bloods, there were differences in the reactions of minor strength so that the number of positive tests was greater with some sera than with others. In the following experiments two sera were selected which behaved identically and gave the smallest number of positive reactions; *i. e.*, the bloods acted upon by these sera reacted positively with all sera. Moreover with the sera chosen there was a distinct break between positive and negative tests, a point of significance for the present issue.

The frequency of the types M+ and M−, and N+ and N−, as already stated, is approximately the same in the four blood groups. According to our present results, there were in 1708 white individuals 326 (19.1 per cent) with negative reactions for M, and in 532 white individuals there were 139 (26.1 per cent) negative for N.

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that the formula $p + q + r = 1$, holds for arbitrarily chosen values, which is obviously not the case.

[5] While this paper was in press, another explanation based upon the assumption of incomplete linkage was proposed by Bauer (*Klin. Woch.*, 1928, vii, 1588).

[6] *Cf.* Morgan (12).

The technique of performing the tests has been described (18). The absorptions and tests for N were made at about 40°C.

It should be stated that the technique offers some difficulties as compared to the common isoagglutination tests. It is necessary to become well acquainted with the method and to know the properties of each serum in order to absorb completely all agglutinins but those in question.

The material for this study was obtained from two maternity clinics in the City of New York. Altogether 166 families were studied; in most of these (122) there were four or more children. Several families were always included in one experiment and also a number of control bloods with known properties.

TABLE IV.

Unions No.	Type of parents	No. of families	No. of children of types		
			M+N+	M+N−	M−N+
1	M+N+ × M+N+	11	31	17	7
2	M+N+ × M−N+	17	40	1	34
3	M+N+ × M+N−	24	60	40	3
4	M+N− × M−N+	5	17	0	1
5	M+N− × M+N−	4	0	17	0
6	M−N+ × M−N+	3 (6)	0	0	18 (29)

The figures in parentheses in unions of type 6, include the three families tested only for M but which, according to our experience, would be of the type M−N+.

In 166 families only the property M was investigated; 64 families were examined for M and N. A representative experiment is given in Table I.

The results for M are summarized in Table II and are arranged in three classes corresponding to the three types of unions and those for N in Table III are similarly arranged.

64 families were examined both for M and N. The results (Table IV) are arranged according to the six sorts of matings and the three types of offspring that have been observed.

A list of the tests is given in Tables V a and V b for the individual families, the former showing the tests for M (102 families) and the latter tests for both M and N (64 families). In each case the children are

TABLE V*a*.**

Reactions for M.

Family No.	Father	Mother	Children				
1	O+	B+	O+ ♂	O+ ♂	O+ ♀	O+ ♀	
2	O+	O+	O− ♀	O− ♂	O+ ♂		
3	AB−	AB+	AB− ♀	B+ ♂	B− ♂	A− ♀	
4	A+	A+	A+ ♂ A+ ♂	A+ ♀	A+ ♀	A− ♂	A− ♂
5	A+	A+	A+ ♀	A+ ♂	O+ ♀	A+ ♂	
6	B−	A+	B+ ♀	B+ ♀	B+ ♂	B+ ♂	
7	O+	O+	O+ ♂	O+ ♀	O+ ♂	O+ ♂	
8	AB+	A+	A+ ♂	B+ ♀	A+ ♂	A+ ♂	
9	A+	O−	A+ ♂ O+ ♀	O+ ♀	A+ ♀	O+ ♂	A+ ♀
10	B+	B+	O− ♂	B− ♀	B+ ♂		
11	O+	AB+	A− ♀	A+ ♂			
12	O+	A+	A+ ♀	O+ ♀	A+ ♀	A+ ♂	
13	O−	O−	O− ♀	O− ♀	O− ♀	O− ♀	
14	AB+	A+	A+ ♂	A+ ♂	A+ ♀	AB+ ♂	A+ ♂
15	AB+	A+	A+ ♀	AB+ ♀	AB+ ♂		
16	B+	O−	B− ♀	O− ♂	O+ ♀	O+ ♂	O− ♂
17	A+	O+	A+ ♂	O+ ♀	A+ ♂	O+ ♂	
18	A+	O+	O+ ♀	A+ ♀	A+ ♂*	A+ ♀*	O+ ♀
19	A+	O−	O+ ♀	O+ ♀	A+ ♀	O+ ♀	
20	A−	B+	O+ ♀	AB+ ♀	AB+ ♀	A− ♀	

* Twins.
** 20 of the 166 families examined were negro families.

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
21	A+	O+	A+ ♀	A+ ♀	O+ ♀	O+ ♂	
22	O+	O+	O+ ♀	O+ ♂			
23	O+	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
24	O+	O—	O+ ♂ O+ ♀	O— ♂ O+ ♀	O— ♂	O+ ♀	O+ ♂
25	B+	A—	AB— ♀ B+ ♀	B+ ♀	B+ ♂	AB+ ♀	AB+ ♂
26	AB+	A+	AB— ♂	A+ ♀	AB+ ♂	B+ ♀	B+ ♂
27	O—	A+	O— ♂	A+ ♀	A— ♂	A— ♂	
28	B+	O+	B+ ♂	O+ ♂	B+ ♀	O+ ♂	
29	O+	A—	A+ ♂	O— ♀	O— ♀	A+ ♀	O— ♀
30	O—	O+	O+ ♀	O— ♀			
31	A+	O—	A— ♀	O— ♀	A+ ♀	A+ ♀	
32	A+	O—	A— ♀	A+ ♀	A+ ♂		
33	O—	O+	O— ♂	O+ ♀	O— ♀	O— ♀	
34	B+	A+	A+ ♂	AB+ ♂	AB+ ♀	AB+ ♀	
35	A—	B+	A— ♀	AB— ♂	B— ♂	AB+ ♂	
36	O—	O+	O+ ♂	O+ ♂	O+ ♂	O+ ♂	O+ ♂
37	O+	A+	A+ ♀	A+ ♂	O+ ♀		
38	A+	A+	A+ ♂	O+ ♂	A+ ♂	O+ ♂	
39	O+	A—	Á+ ♀	O+ ♂	O+ ♂		
40	O+	A+	A+ ♀	A+ ♀	A+ ♂	A+ ♂	A+ ♀
41	A+	B+	AB+ ♀ AB+ ♂	AB+ ♂	A+ ♀	AB+ ♀	AB+ ♂

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
42	A+	B−	A+ ♂	A− ♀	AB+ ♀	A− ♂	
43	A+	B+	A+ ♀	A+ ♂	AB+ ♀	A+ ♀	
44	B+	A+	A+ ♀	A+ ♂	A+ ♀		
45	B+	O−	O− ♂	B+ ♂			
46	AB−	O+	B+ ♀	B+ ♀*	B+ ♀*	B+ ♀	
47	A+	O+	O+ ♂	A+ ♀	O+ ♂	A+ ♂	O+ ♂
48	A+	A+	O+ ♂	O− ♂	O− ♂		
49	A+	A+	A+ ♀	A+ ♀	O− ♀	A− ♀	
50	B+	A+	B− ♀	O+ ♂	AB+ ♀		
51	O+	O−	O+ ♀	O+ ♀*	O+ ♀*	O+ ♀	
52	O+	O−	O+ ♂	O− ♀	O+ ♂	O− ♂	O− ♂
53	A+	B−	A+ ♀	AB+ ♂	AB− ♂	A− ♂	
54	A+	A+	A+ ♀	A+ ♂	A+ ♀		
55	A+	A+	A+ ♂	A+ ♀			
56	O+	A+	O+ ♂	A+ ♀	O+ ♀		
57	O−	O+	O+ ♀	O+ ♂	O+ ♀	O+ ♂	
58	O+	A+	A+ ♀	A+ ♂	A+ ♀		
59	O+	A+	O+ ♂	O− ♂	O+ ♂	O+ ♀	A+ ♂
60	B−	O−	O− ♂	O− ♂	O− ♀		
61	O+	A−	O+ ♀	O+ ♂	A+ ♀		
62	A+	A+	A+ ♀	O+ ♂	A+ ♂	O+ ♀	
63	O+	A+	O+ ♀	O+ ♂	A+ ♂	A+ ♂	A− ♂

* Twins.

TABLE V*a*—*Continued.*

Family No.	Father	Mother	Children				
64	O+	O+	O+ ♂ O+ ♂	O+ ♂	O+ ♀	O+ ♀	O+ ♂
65	O+	O−	O+ ♂	O+ ♀			
66	O+	B+	B+ ♂	B+ ♂			
67	A+	O+	A+ ♂	O+ ♂			
68	O+	O+	O+ ♀	O− ♂	O+ ♀	O− ♀	
69	A+	O+	O+ ♀	O+ ♀	A+ ♀	O− ♀	A+ ♂
70	A+	O+	O+ ♀ A+ ♂	A+ ♂ O+ ♂	O+ ♀ O+ ♂	O+ ♀ A+ ♀	O+ ♀
71	AB−	O+	O+ ♀ O+ ♀	A+ ♀	B+ ♂	A+ ♂	A+ ♀
72	B+	A−	O+ ♀	O+ ♀	B− ♀	AB+ ♀	
73	A+	O+	O+ ♀	O+ ♀	A− ♂	O+ ♂	
74	O+	A+	A+ ♀	A+ ♂	O+ ♂	O+ ♂	
75	B+	O+	O+ ♂	O+ ♂	B+ ♂	O+ ♀	
76	O+	A+	O+ ♂ O+ ♂	O+ ♂	A+ ♀	A+ ♂	A+ ♀
77	O−	B+	O+ ♂ O+ ♀	O+ ♀	O+ ♂	B+ ♀	O+ ♂
78	O+	O+	O+ ♀ O+ ♀	O+ ♂ O+ ♀	O+ ♂ O+ ♂	O+ ♀	O− ♀
79	O+	B+	B+ ♂	B+ ♂	B+ ♂		
80	O+	B+	O+ ♂	B+ ♀	O+ ♂	B+ ♂	O+ ♂
81	O−	B+	O+ ♀	O+ ♂	B+ ♂		
82	O+	O+	O+ ♂	O+ ♂	O+ ♂		

TABLE V*a—Concluded.*

Family No.	Father	Mother	Children				
83	O+	O+	O+ ♂	O− ♀	O+ ♀		
84	O+	O+	O− ♀	O− ♂	O+ ♂	O+ ♂	.
85	O+	B−	B− ♂	B+ ♂	B− ♂		
86	A+	B+	B+ ♀	O+ ♀	AB+ ♀		
87	O+	A+	O+ ♀ O+ ♀	A+ ♀	O+ ♂	O− ♀	A+ ♂
88	O+	O−	O+ ♂	O+ ♀	O+ ♂		
89	A+ .	O−	A+ ♂ O+ ♀	O+ ♂	O+ ♂	A+ ♂	O+ ♂
90	AB−	O+	A− ♂	B− ♀	A+ ♂		
91	O−	AB+	A− ♂ B− ♀	B− ♀	A− ♀	B− ♀	A+ ♀
92	O+	O+	O+ ♀	O+ ♂	O+ ♂	O+ ♂	
93	B+	B+	B+ ♂	B+ ♂	B+ ♂	B+ ♂	
94	O+	A−	A+ ♀	A− ♂	A+ ♂	A− ♂	A+ ♀
95	B+	A+	B+ ♀	B+ ♂	O+ ♂	A+ ♀	
96	A+	A−	O− ♂ O− ♂	A− ♀	A+ ♂	A+ ♀	O− ♂
97	A+	O+	A+ ♂ O+ ♂	A+ ♀	O+ ♀	O+ ♀	O+ ♂
98	O+	A+	O+ ♀	O+ ♀	O+ ♀	O+ ♂	O+ ♀
99	B+	O+	B+ ♂ O+ ♂	B+ ♀	O+ ♀	O+ ♀	O+ ♂
100	A+	A+	A+ ♂	A+ ♂	A+ ♂	A+ ♀	
101	B+	O+	O+ ♂ B+ ♀	B+ ♂ O+ ♀	B+ ♀	O+ ♂	O+ ♂
102	B−	A−	A− ♀	A− ♀	A− ♂	AB− ♀	

TABLE V*b*.

Reactions for M and N.

Family No.	Father	Mother	Children			
103	O++	A++	A++ ♂ O+− ♀ A−+ ♂ O++ ♂ A+− ♂			
104	O−+	A++	O−+ ♀ O++ ♂ O++ ♂ A−+ ♂			
105	O−+	O−+	O−+ ♀ O−+ ♂ O−+ ♂ O−+ ♂			
106	O++	AB+−	B++ ♂ B++ ♀			
107	O+−	A++	O+− ♀ A++ ♂ A+− ♀ A+− ♀			
108	O+−	A+−	O+− ♀ A+− ♀ O+− ♂ O+− ♂			
109	O+−	A++	A++ ♂ O++ ♂ A+− ♂ A+− ♂ O−+ ♂			
110	A++	O−+	A++ ♀ O++ ♀ O++ ♂ O++ ♀			
111	A+−	A−+	A++ ♀ A++ ♂ A++ ♀ A++ ♂ A++ ♂			
112	O++	O+−	O++ ♂ O++ ♀ O++ ♀ O+− ♂ O++ ♂			
113	A−+	A++	A++ ♂ A++ ♀ A−+ ♀			
114	A++	O++	A−+ ♀ O+− ♂ O−+ ♀ A++ ♀ O+− ♂ A++ ♂			
115	A+−	A++	A++ ♂ A++ ♀ A++ ♀ A++ ♂			
116	O++	B−+	B−+ ♀ O−+ ♀ B−+ ♂ O−+ ♂ O++ ♂ B++ ♀			
117	O−+	O++	O++ ♀ O−+ ♀ O−+ ♀ O++ ♂ O++ ♂ O++ ♂			
118	O+−	A++	O++ ♀ O++ ♂ O++ ♂ O+− ♀ A+− ♀ A++ ♀ A+− ♀ O++ ♀ A++ ♀			
119	O−+	A++	A−+ ♂ A−+ ♀ A++ ♀ O−+ ♀ A++ ♂ A++ ♀ O++ ♀			

TABLE V*b—Continued.*

Family No.	Father	Mother	Children			
120	A++	O−+	A++ ♂	A++ ♀	A++ ♂	A++ ♀
121	B++	A++	AB−+ ♂ A+− ♂	A++ ♂ A++ ♀	A++ ♀	A++ ♂
122	O+−	A++	O++ ♂ A++ ♂	A+− ♂	A+− ♂	A++ ♀
123	O−+	A+−	A++ ♂	A++ ♀		
124	A+−	A++	A++ ♂ A+− ♀	O+− ♀	A++ ♀	O+− ♂
125	O++	A++	A+− ♂ O++ ♀	A++ ♀	O++ ♀	A++ ♂
126	B++	B++	B++ ♀	B+− ♂	O++ ♂	
127	A+−	B++	B−+ ♂	A+− ♀	O+− ♂	
128	O+−	O++	O+− ♀	O++ ♀	O+− ♀	O++ ♂
129	O++	O+−	O+− ♂ O+− ♀	O+− ♂ O++ ♂	O++ ♀	O++ ♀
130	A−+	A−+	A−+ ♂ A−+ ♂	O−+ ♂ O−+ ♂	A−+ ♀	A−+ ♀
131	A+−	O−+	O++ ♀	O−+ ♀	O++ ♂	O++ ♂
132	A−+	O++	A++ ♀ O++ ♂	O−+ ♀	O−+ ♂	O++ ♂
133	O++	O+−	O+− ♂ O+− ♀	O++ ♀	O+− ♂	O+− ♀
134	O+−	A+−	A+− ♀	A+− ♀	A+− ♂	
135	O+−	O++	O++ ♀	O+− ♂	O++ ♀	
136	A++	A−+	A−+ ♂ O−+ ♀	A−+ ♂	A−+ ♀	A−+ ♀
137	B+−	O++	B++ ♂	B+− ♀		

TABLE V*b*—*Continued.*

Family No.	Father	Mother	Children			
138	A+−	O++	O++ ♀	A+− ♀		
139	A+−	B−+	B++ ♀	B++ ♀	B++ ♀	AB++ ♀
140	A++	B++	O++ ♀	O++ ♂	A++ ♂	AB+− ♂
			O++ ♀	O++ ♂		
141	O++	B++	B++ ♀	B++ ♂	O++ ♀	O−+ ♂
			O++ ♂			
142	O+−	O++	O++ ♂	O++ ♂	O++ ♀	O++ ♂
			O−+ ♂			
143	A+−	A+−	A+− ♂	A+− ♀	A+− ♂	A+− ♂
144	O++	O++	O++ ♀	O++ ♀	O+− ♀	O++ ♂
145	O−+	A++	A++ ♂	A++ ♂	A−+ ♀	A++ ♀
146	O+−	A++	O++ ♀	A++ ♂	A+− ♂	O+− ♂
147	A−+	O++	A−+ ♀	A++ ♂	O−+ ♀	A−+ ♂
148	A++	B−+	B−+ ♀	A++ ♂		
149	O−+	B−+	B−+ ♂	O−+ ♀	O−+ ♀	B−+ ♂
			O−+ ♂	B−+ ♂	O−+ ♂	B−+ ♀
150	O−+	A++	A−+ ♀	A++ ♀	A+− ♂	A−+ ♀
151	B+−	O−+	O÷÷ ♀	B÷+ ♀	B÷+ ♀	
152	O−+	O++	O−+ ♂	O++ ♂		
153	A−+	O++	A++ ♂	A−+ ♀	O−+ ♂	O−+ ♂
			O++ ♀	O−+ ♀		
154	A++	B+−	O÷÷ ♀	B++ ♀	O÷− ♂	
155	A+−	A++	A++ ♂	A+− ♂	A++ ♂	A÷− ♂
			A÷− ♂	A++ ♂		
156	A++	O++	O÷÷ ♀	O÷− ♀	O÷− ♂	A+− ♀

TABLE V*b*—*Concluded.*

Family No.	Father	Mother	Children			
157	O−+	O++	O++ ♀ O++ ♂ O−+ ♀ O−+ ♀			
			O++ ♀ O−+ ♂			
158	O++	A−+	A++ ♀ A++ ♂ O++ ♂			
159	O++	B+−	B++ ♀ O++ ♀ B++ ♀ B++ ♂			
160	A++	O++	A+− ♂ A++ ♀ O+− ♂ A−+ ♂			
			O++ ♂ O+− ♀			
161	O++	A++	A++ ♀ A++ ♀ A+− ♀ A+− ♂			
			A−+ ♀			
162	A+−	O++	A++ ♂ A++ ♂ A+− ♀ A+− ♂			
			O++ ♀			
163	B+−	O+−	O+− ♂ B+− ♂ O+− ♂ B+− ♂			
			O+− ♂ B+− ♂			
164	B+−	A++	O++ ♀ A++ ♂ A++ ♂ O++ ♂			
			AB++ ♂			
165	B+−	O++	O++ ♀ O+− ♀ B+− ♀ O+− ♀			
166	B+−	O++	B++ ♀ O++ ♀ B+− ♂			

recorded in order of decreasing age beginning with the eldest. The letters designate the groups, and the signs + and − the reactions for M (Table V*a*). In Table V*b* the first + or − sign designates the test for M and the second sign that for N.

As to the heredity of the factors A and B our results agree with the established fact that they are inherited as Mendelian dominants, except for three families in which A or B appeared in children when they were absent in the blood of the parents. These cases were considered as instances of illegitimacy and were excluded from the tabulations. One of these families was examined only for M and two for both M and N. The results were:

Father	Mother	Children			
O+	O+	O+ ♂	B+ ♂	O+ ♀	A+ ♀
O++	O++	A++ ♀	A++ ♀	O++ ♀	O+− ♀
O+−	B−+	AB++ ♀	O+− ♂	O++ ♀	O++ ♀

In family 71, AB × O, there were two children in group O; the mother refused reexamination.

From the data reported it is evident that the agglutinogens studied are inherited properties. If we consider M and N separately they would seem to behave like Mendelian dominants. The characters cannot be recessive since in unions + × + there are children whose blood lacks the property. This result is to be expected if there are individuals among the parents heterozygous for a dominant character. If the absence of the agglutinogens is recessive there should occur no positive reactions in children from unions − × −. This is actually borne out by the observations. Thus in six such families with M − parents all the children (29 in number) gave negative reactions for M; likewise in the four families with N − parents all the children (17 in number) belonged to the N − type. In this respect our findings are analogous to the rule established by von Dungern and Hirschfeld for the isoagglutinogens A and B, *i. e.*, that these do not appear in the offspring if they are absent in both parents.

In order to discuss the numerical results for M alone in the three sorts of matings it is necessary to know the incidence of homozygous (MM) and heterozygous (Mm) individuals among the M+ parents. From a formula quoted by Johannsen (19), the following values are obtained: MM = 29.6; Mm = 49.6; mm = 20.8 (approximately 30, 50, 20, respectively). According to this formula the percentage of homozygous individuals equals $100 - 20 \sqrt{R} + R$, that of heterozygous $20 \sqrt{R} - 2R$; where R is the percentage of recessive (M −) individuals observed in the population. The figures of M+ and M − reactions are taken from all individuals, including the parents, of the 166 families examined for M.

Calculating from these figures the number of M − children in the unions M+ × M+ one has to consider only those in which both parents are heterozygous, *i. e.*, approximately 5/8 × 5/8; since 1/4 of the children of these matings should be M −, 9.8 per cent of M − children are to be expected; the observed value is 7.6 per cent.

In the unions M+ × M − 50 per cent of the offspring of heterozygous M+ parents may be expected to be M −; *i. e.*, 5/8 × 1/2 = 31.3 per cent; actually 34 per cent M − children were found.

In the smaller series where both factors were examined (see Table IV) the agreement between the calculated and the observed values is not so satisfactory.

Applying the formula of Johannsen for the factor N we obtain NN = 23.1; Nn = 49.8; nn = 27.1. Calculated as above the figures are in matings N+ × N+ 11.7 per cent N− children (observed 11.5 per cent) and in matings N+ × N− 34.2 per cent N− children (observed 33.1 per cent).

So far the cursory analysis of the results does not contradict the idea of two independent factors. However, there is evidence which does not seem compatible with this view. In the first place, if the factors were independent one would expect a certain, although small, percentage of bloods to lack both M and N, that is, if the genotype M − N − is not lethal, or its phenotype indistinguishable from one of the other types. In fact, as has been stated formerly (18) no such case has been found in the examination of more than 1200 specimens[7] and in each M − blood the reaction for N was found to be very strong. Further evidence emerges from an analysis of results in families examined for both properties. One sees that the frequency of M − children in the three sorts of matings, Nos. 1, 3, and 5 (Table IV) of parents M +, varies greatly according to the N reactions of the parents and that likewise the occurrence of children N − in matings 1, 2, and 6 is influenced by the presence or absence of M in the parents. A similar statement holds for the appearance of M − or N − children in matings 2 and 4, and 3 and 4, respectively.

Actually in most of the six sorts of unions the observed figures do not tally satisfactorily with those to be expected on the basis of two independent factors if one computes the expectancy from the figures given above for heterozygous and homozygous individuals. Thus in matings 2 and 3 there are too many children of the type M − N + or M + N −, respectively, and in union 4 there appear with one exception only children of type M + N +. These numerical results could be interpreted in various ways. One hypothesis consists in assuming two genes which, when homozygous, would determine the phenotype M + N − and M − N + respectively, while the phenotype M + N +

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[7] This number includes the blood of negroes and Indians. About 900 of these were tested with the improved technique, namely, performing the tests for N at about 37–40°C.

would correspond to the heterozygous gene constitution. This view accounts for the non-existence of the type $M-N-$. On the basis of this hypothesis the expected values for the types of offspring are: mating 1: $M+N+$ 50 per cent, $M+N-$ 25 per cent, $M-N+$ 25 per cent; mating 2: $M+N+$ 50 per cent, $M-N+$ 50 per cent; mating 3: $M+N+$ 50 per cent, $M+N-$ 50 per cent; mating 4: $M+N+$ 100 per cent; mating 5: $M+N-$ 100 per cent; mating 6: $M-N+$ 100 per cent.[8] Allowing for the relatively small number of individuals examined these figures agree fairly with those observed and better than the figures calculated for independent factors. Especially striking is the fact that in matings 2 and 3 there are almost no children $M+N-$ or $M-N+$, respectively, and in mating 4 only one child not of the type $M+N+$. Still, there are five cases which contradict the hypothesis mentioned, namely, the individual $M+N-$ in union 2, the three children $M-N+$ in union 3, and one child $M-N+$ in union 4. To attribute all these five exceptions to illegitimacy seems hazardous since only a proportion of illegitimate children would be detected by the tests employed and because the number of the exceptional cases is high compared with that of children which do not conform to the rule of von Dungern and Hirschfeld.

It may be pointed out that in all of the five exceptional cases in unions 2, 3, and 4, the father and not the mother is of the type opposite to that of the child, *e. g.*, father $+-$, child $-+$.

On the basis of the assumption just discussed and with the aid of the formula used above, the frequency of one type could be used to calculate the frequency of the other two types. Starting from the figure 20.8 for $M-$ in a certain population the computation gave the value of 29.6 $N-$; similarly in a population of 205 Indians examined by us, the observed value of 4.9 $M-$ leads to an expectancy of 60.7 for $N-$. Both these figures are in good agreement with those observed, namely, 26.1 and 60.0 respectively.[9]

An alternative hypothesis would suppose a close linkage between M and N. If, then, the gene combinations Mn and mN are numerically predominant this could explain the observed figures and also the occurrence of the exceptional cases aforementioned, but unless one assumes a lethal effect there arises a difficulty from the following con-

[8] The numbers of the matings refer to those given in Table IV.
[9] These results will be discussed in another publication (20).

sideration. If the factors M and N have been in the race for a long time the occurrence of cross-overs should by now have reduced the assumed numerical difference. However, on account of the existence of agglutinogens similar to M and N in anthropoids (chimpanzees) (18) it does not seem likely that they are due to recent mutations.

If in the unions listed as No. 1, Table IV, one of the parents be homozygous with respect to both M and N, then all children of such a union would be of the phenotype M+N+. The fact that this was not the case in any of the eleven families shows that none of the parents can be homozygous for both M and N, but such homozygous parents may have occurred in unions 2 and 3.

In view of the limited number of families studied it would seem premature to attempt a final interpretation and to discuss further possibilities such as the existence of more than two allelomorphs. Also it has to be considered that the state of affairs may be complicated, e. g., by interacting or modifying effects of factors determining hitherto unknown agglutinable structures.

It may be added that there is no indication of a linkage between M and N and the isoagglutinogens A and B, and, as in the case of A and B, no evidence of a sex linkage.

SUMMARY.

The heredity of two agglutinable structures demonstrable by immune agglutinins was studied in 166 families. From the data collected it is evident that one deals with a case of Mendelian inheritance. The main result of the studies is the demonstration that it is feasible to investigate the heredity of serological structures of human blood other than the group agglutinogens. Irrespective of the ultimate theory it seems very probable that the properties M and N do not appear in the offspring when they are absent in both parents—a conclusion substantiated by the examination of ten families with 46 children. These findings offer the prospect of forensic application to cases of disputed paternity and, in our opinion, a correct decision could already be given, at least with great probability, provided the reagents are available and the method properly applied. Of course further work is needed before the test can be adopted as a routine procedure.

For the material used in this study we express our thanks to Dr. Harold Bailey and Dr. H. C. Williamson and also to Miss M. C. Skelly, Superintendent of the John E. Berwind Free Maternity Clinic, and to Miss Agnes Martin, Superintendent of the Manhattan Maternity Hospital.

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VARIANTS OF HEMOLYTIC STREPTOCOCCI; THEIR RELATION TO TYPE-SPECIFIC SUBSTANCE, VIRULENCE, AND TOXIN.

By E. W. TODD,* M.D., and R. C. LANCEFIELD, Ph.D

(From the Hospital of The Rockefeller Institute for Medical Research.)

In previous communications one of us (1) described three substances which can be extracted from hemolytic streptococci: (1) *The nucleoprotein P* is common to all strains of hemolytic streptococci and is serologically related to the nucleoproteins of pneumococci and of green streptococci. (2) *The non-protein substance C*, which appears to be a carbohydrate, is found in all strains of hemolytic streptococci but is species-specific and serologically distinct from the carbohydrate fractions of pneumococci and green streptococci. (3) *The type-specific fraction M*, which is probably protein in nature, has not been isolated from other species of microorganisms; it occurs in serologically distinguishable forms which serve to differentiate hemolytic streptococci into types.

One of us (2) has previously described two forms of hemolytic streptococci distinguishable by the morphology of their colonies. The general appearances of these colonies, when grown on a special medium and viewed by reflected light, are the same as those which distinguish the rough and smooth varieties of other bacteria but the terms "R" and "S" have not been used and the colonies have been designated "matt" and "glossy" to avoid confusion which would otherwise certainly arise from the circumstance that the rough, or matt, colonies are the virulent type while the smooth, or glossy, forms are relatively avirulent. It is the purpose of this paper to show that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies.

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* British Medical Research Council Fellow.

It will also be shown that filtrates from both matt and glossy cultures of hemolytic streptococci contain skin-reactive toxin.

Some Characteristics of the Type-Specific Substance M.

The type-specific substance M is prepared by a modification of Porges' (3) method. The bacterial bodies are extracted with N/20 HCl in salt solution at the temperature of boiling water; and after neutralization and clarification by centrifuging the resulting clear slightly yellow fluid is used as an antigen for precipitin reactions. Bacterial extracts prepared in this manner contain the type-specific substance M and in addition they also contain small quantities of the non-type-specific fractions, P and C, which may cause some precipitation with sera prepared against any strain of hemolytic streptococcus. To avoid the appearance of these confusing precipitates the serum may be absorbed with any heterologous strain of hemolytic streptococcus and by this means a specific anti-M serum is obtained which will only precipitate in the presence of the homologous M substance.

In the original work (1) which led to the recognition of the type-specific substance M thirteen strains were used which Dochez, Avery and Lancefield (4) had classified, some years earlier, into types by agglutination and protection tests. Ten of these strains yielded type-specific precipitating substances which differentiated them into types corresponding to those originally determined. The remaining three strains failed to yield any type-specific substance although they had been classified by agglutination and protection in the earlier work. It was suggested that prolonged cultivation in the laboratory had caused these three strains to lose their type-specific characteristics. In the present communication it will be shown that matt cultures containing the type-specific substance can, by various means, be reduced to the glossy form in which the type-specific substance is no longer present.

The Preparation of Anti-M Serum.

As the type-specific substance M, after separation from the bacterial cell, does not produce any demonstrable antibody, when injected into animals, the only antigen available for preparing anti-M serum

is a suspension of bacteria in the matt form. Matt cocci contain the three substances P, C and M and sera prepared with these organisms, therefore, contain antibodies to each of the three substances. On the other hand, cocci in the glossy form contain the two substances P and C but are devoid of the type-specific substance M; consequently, antibacterial sera prepared with glossy strains contain antibodies to P and C but do not contain any type-specific antibody.

Anti-M sera were prepared by inoculating rabbits intravenously with 16 hour cultures of matt cocci grown in tryptic broth. Immunization was commenced with four injections on consecutive days of 1 cc. of heat-killed culture, followed a week later by four doses of 2 cc. of the same vaccine. During the 3rd week the rabbits received four doses of 0.5 cc. of living culture and in the 4th week this dose was doubled. A final series of doses of 2 cc. of living culture was given during the 5th week and the animals were bled 10 days after the last injection. The sera of animals immunized in this way usually contained a satisfactory quantity of antibody to the type-specific substance M but with some strains it was necessary to continue immunization with 5 cc. and even 10 cc. of living culture before useful sera could be secured. A few strains have been encountered which, although they were in the matt form and moderately virulent for mice (0.001 cc. or 0.0001 cc.), produced only traces of type-specific antibody in rabbits even after intensive immunization. Twelve rats were immunized with a strain (New York V E14) which had previously failed to produce more than traces of type-specific antibody in the sera of twelve immunized rabbits. The rats, which remained perfectly well during immunization, received the following intraperitoneal doses: 1st week 3 doses of 0.25 cc. of heat-killed culture; 2nd week 4 doses of 0.5 cc. of heat-killed culture; 3rd week 4 doses of 1.0 cc. of heat-killed culture; 4th week 3 doses of 1.0 cc. of living culture; 5th week 4 doses of 2.0 cc. of living culture; 6th week 4 doses of 2.0 cc. of living culture. Seven of the immunized rats yielded moderately good anti-M sera; the remaining five sera contained traces of type-specific antibody. As rats appear to be able to tolerate relatively larger doses of culture than rabbits it is possible that they may be more suitable for the preparation of anti-M serum; but the small yield of serum makes this method impracticable for routine purposes.

The Absence of the Type-Specific Substance M from Organisms Which Form Glossy Colonies.

It has already been stated that the type-specific substance M is found in HCl extracts of matt hemolytic streptococci and that it is not found in similar extracts prepared from the glossy variants.

This is demonstrated by the following experiment:

Four type-specific anti-M sera were prepared by immunizing rabbits with four matt strains of hemolytic streptococci belonging to different serological types and by subsequently removing the non-type-specific antibodies from the sera by absorption with heterologous strains.

Table I gives the precipitin reactions of the four sera with HCl extracts prepared from cultures of the homologous cocci (1) in the matt form and (2) in the glossy variant form.

TABLE I.

Precipitin Reactions of Type-Specific Anti-M Sera with Extracts of the Homologous Strains (1) in the Matt Form, (2) in the Glossy Form.

Volumes of extracts*	Strain S43		Strain S23		Strain C203		Strain London	
	1	2	1	2	1	2	1	2
	Extract from matt form	Extract from glossy form	Extract from matt form	Extract from glossy form	Extract from matt form	Extract from glossy form	Extract from matt form	Extract from glossy form
cc.								
0.4	+++	−	++++	−	++	±	++	−
0.1	++	−	++	−	++±	−	++±	−
0.025	+	−	+	−	++	−	+±	−

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

It will be seen from Table I that the extract prepared from the matt form of each strain gave a good precipitin reaction with the homologous antiserum; on the other hand, the extract prepared from the glossy form of each strain gave a negative precipitin reaction with the single exception of Strain C203 which gave a faintly positive reaction. Although this experiment seems to show that each of the four strains lost its type-specific substance in the process of degradation to the glossy variant form, yet it will be seen from the results of experiments with highly concentrated extracts that, in reality, only one strain had completely lost its type-specific substance. Highly concentrated extracts were prepared from each of the four glossy variants referred to in Table I in the following manner: The centrifuged deposit from 9 liters of broth culture was extracted with HCl; and, after concentration by alcoholic precipitation, the precipitate was redissolved in

5 cc. of salt solution. Precipitin tests, with the concentrated extract prepared from Strain S23 were negative showing that this strain was completely devoid of type-specific substance. Precipitin tests, with the other three concentrated extracts and their homologous specific anti-M sera, were weakly positive showing that three of the glossy cultures retained traces of type-specific substance. The minute amounts of specific substance remaining in these cultures can be judged from the following figures—9 liters of broth culture were used in preparing extracts from the glossy forms—50 cc. of broth culture were used in preparing extracts from the matt forms—180 times more culture was, therefore, used in the preparation of the glossy extracts than in the preparation of the matt extracts and, in spite of these disproportionate quantities, the latter extracts contained the larger quantity of the type-specific substance M. It appears from these experiments that hemolytic streptococci are rarely degraded to the point at which type-specific substance completely disappears.

Some Characteristics of Matt Cultures of Hemolytic Streptococci.

Twenty-eight strains of hemolytic streptococci were examined immediately after isolation from pathological conditions in the human body. The sources of these cultures included cases of puerperal septicemia, pleural effusion, scarlet fever, pneumonia and sinusitis; strains were also isolated from the depths of enucleated tonsils and from throat swabs. In twenty-one cases the cultures when freshly isolated, were entirely composed of matt colonies; in five cases both matt and glossy colonies were seen on the plates; and in two cases the cultures were entirely glossy, but as both the glossy strains were obtained from throat swabs and were accompanied by other bacteria there was no evidence that the hemolytic streptococci were playing a pathogenic rôle. Table II gives the source and character of the cultures.

There is, therefore, some evidence that cultures freshly isolated from human sources are usually of the matt variety and this statement particularly applies to diseases such as septicemia in which the streptococci are the undoubted causal agent.

It is frequently found that matt strains of hemolytic streptococci, isolated from human lesions and undoubtedly pathogenic for man, are

TABLE II.

The Morphological Appearance of Colonies of Freshly Isolated Strains of Hemolytic Streptococci and the Source of the Cultures.

No.	Disease	Source of culture	Pure culture or mixed flora	Morphology of colonies
1	Puerperal septicemia	Blood culture	Pure culture	Matt
2	"	"	"	"
3	"	"	"	"
4	Nephritis and septicemia	"	"	"
5	Endocarditis	Postmortem culture from spleen	"	"
6	Pneumonia and pleural effusion	Chest fluid	"	"
7	Sinusitis	Nasal mucus	"	"
8	Scarlet fever	Throat swab	Almost pure culture	"
9	" "	"	"	"
10	Tonsillitis	"	"	"
11	Enlarged tonsils	From depths of enucleated tonsils	"	"
12	' "	"	"	"
13	" "	"	"	"
14	" "	"	"	"
15	Pharyngitis	Swab from pharynx	"	"
16	Sinusitis	" nose	"	"
17	Tonsillitis	" tonsils	"	"
18	"	" "	"	"
19	Pneumonia	Sputum	Chiefly S. *hæmolyticus; B. influenzæ*	"
20	" (Type IV pneumococcus)	"	Mixed flora	"
21	" "	"	Equal numbers hemolytic streptococci and pneumococci	"

22	Pneumonia (Type IV pneumococcus)	Sputum	Mixed flora	Matt and glossy
23	Enlarged tonsils	From depths of enucleated tonsils	Pure culture	" "
24	"	"	Almost pure culture	" " "
25	"	"	" "	" " "
26	"	"	" "	" " "
27	Tonsillitis	Swab from tonsils	Mixed flora, about 5 per cent hemolytic streptococci	Glossy
28	Pneumonia (Type IV pneumococcus)	" " "	Mixed flora	"

avirulent for mice (M.L.D. 0.5 cc. or 1.0 cc.).   Such a culture en-
tirely composed of matt colonies will be referred to in this paper as the
matt attenuated form because it is avirulent for mice yet possesses
the colony characteristics and the specific substance of the virulent
form.   Attempts to increase the virulence of matt attenuated cul-
tures, by mouse passage, have always been successful although some
of the strains tested have required very many passages before the
maximal virulence of 0.000001 cc. has been attained and in some
cases the virulence has never risen above 0.0001 cc. even after 80 or
90 consecutive passages through mice.

Virulence is the only quality which distinguishes the matt virulent
form from the matt attenuated variety as these colonies are identical
in appearance, and serological examination of HCl extracts does not
show any significant difference in the quantity of type-specific sub-
stance which can be extracted from equal volumes of the two cultures.
From these experiments it appears that the matt form, which is
always potentially virulent, may occur in, at least, two separate
varieties characterized by quantitative differences in virulence for
mice; and it is probable that, by suitable passage experiments, addi-
tional forms can be obtained distinguishable by different degrees of
virulence for other species of animals.

Methods of Converting the Matt Form to the Glossy Variant.

The degree of ease with which glossy variants can be obtained from
different strains varies enormously.   In some cases great difficulty
is experienced in maintaining laboratory stock cultures of the matt,
or potentially virulent, form as they spontaneously change to the
glossy variant even when stored in blood broth in the ice box.   In
these cases it is necessary to resort to frequent mouse passages to
prevent the total loss of the matt form.   On the other hand, matt
strains have been encountered which do not show any tendency to
change to the glossy variant after repeated subcultivations on agar.
Intermediate between these extremes are strains which develop a
small proportion of glossy colonies after repeated subcultivations on
agar.   When one of the glossy colonies derived from these strains is
subcultured in broth and replated on agar a mixture of matt and
glossy colonies usually appears but occasionally a pure glossy culture

may be obtained by this method. By repeated selection of glossy colonies and subcultivation in broth a culture composed entirely of glossy colonies can often be obtained and in some instances a pure culture of the glossy variant can be secured by the simple process of repeated subcultivation on agar slants.

Griffith (5) and others (6–8) have shown that smooth pneumococci can be converted to the rough form by cultivation in the homologous anti-S serum. We have applied this technique to hemolytic streptococci and have found that the cultivation of matt strains in undiluted homologous anti-M serum of high titer is the quickest and most reliable method of obtaining glossy variants, and with some highly stable matt strains this is the only method by which we have been able to secure the glossy form. Here again there are wide differences between individual strains; in some cases, a few transfers in serum suffice to convert a virulent matt culture, containing abundant M substance, into the glossy avirulent variant devoid of any specific substance; in other cases, after as many as 90 transfers in high titer serum traces of the type-specific substance M can still be detected in concentrated bacterial extracts, although the colonies appear to be glossy and the organisms have lost their virulence for mice. Attempts have been made to rid these cultures of the remaining traces of type-specific substance by alternately cultivating the cocci in immune serum, plating out, selecting the most glossy colonies and again subculturing in immune serum; one strain was subjected to this treatment thirty times after 90 previous consecutive transfers in immune serum but at the end of the experiment it still retained traces of the specific substance.

Some Characteristics of Glossy Cultures of Hemolytic Streptococci.

The glossy variant is avirulent for mice in comparison with the matt virulent culture from which it is derived and attempts to raise the virulence of the variants by mouse passage have usually been unsuccessful. It is, however, possible to obtain glossy cultures which are partially virulent for mice. A strain which, in the matt virulent form, killed mice regularly in doses of 0.000001 cc. or 0.0000001 cc. was cultivated in the homologous anti-M serum and, after 55 transfers in 50 per cent serum, a pure culture of the glossy variant was ob-

tained which did not contain any type-specific substance, yet this glossy culture was sufficiently virulent to kill mice regularly in doses of 0.01 cc. and occasionally in doses of 0.001 cc. More prolonged cultivation in anti-M serum did not cause any further decrease in virulence. The partially virulent glossy culture was passed through twenty-five mice intraperitoneally but the virulence remained unchanged and there was no reappearance of type-specific substance. This appears to be an exceptional strain as the M.L.D. of the majority of glossy strains is 0.5 cc. or 1.0 cc.

During the process of conversion from matt to glossy various types of colonies appear, which may possibly represent intermediate forms or may be due to individual colonies containing a mixture of matt and glossy cocci. We have observed that different strains sometimes show peculiarities in the morphology of their colonies which are so striking that the strain can be recognized either in the matt or glossy form. In addition to these strain peculiarities other varieties of colonies appear during the gradual change from matt to glossy. Griffith (9) has noted that in spite of the apparently diverse appearances of streptococcal colonies three forms can generally be distinguished. Two of his forms appear to correspond to our matt and glossy colonies and the third is characterized by a soft consistency, a whitish opaque raised center and a thin translucent margin. In a previous communication one of us (10) described a similar form of colony, which differed in the important respect of being tough instead of watery; but further observation has shown that colonies characterized by a flat marginal zone surrounding a central eminence may occur in two forms, corresponding to the matt or to the glossy state. The matt form of this colony is opaque and of tough consistency with a central eminence surrounded by a flat marginal zone; the glossy form has a similar contour but is soft and watery. The irregular shape of these colonies causes difficulty in observing the light-reflecting character of their surfaces but the matt and glossy forms can generally be distinguished by other characteristics. These observations seem to indicate that this third type of colony is not a distinct entity separate from the matt and glossy forms; but we have failed to determine the significance of these very characteristic colonies.

The classification of colonies is further complicated by the occasional appearance of pseudoglossy forms. When a matt culture is spread on a plate the colonies in close proximity to each other may present the typical matt appearance but widely separated colonies in the same culture may be glossy in appearance. If one of the latter colonies is selected and spread on a fresh plate a pure culture of typical matt colonies may result. Pseudoglossy colonies are generally larger than true matt or true glossy colonies but they so nearly resemble the true glossy form that they are liable to cause confusion.

Owing to these variations in the appearance of colonies we have been unable to rely entirely on the colony form as a guide to the character of cultures. The criteria we have used to determine when a culture is completely degraded from the matt state are: (1) that concentrated HCl extracts of glossy cultures should not cause any precipitation when mixed with pure homologous anti-M serum (absorbed with a heterologous strain to remove the antibodies to P and C); (2) that the result of the above test should remain unchanged after the culture has been passed through a mouse.

It will be seen in the detailed description of experiments that we have only been able to secure one strain in this completely degraded state.

Reversion of Glossy Cultures to the Matt Form.

Dawson and Avery (11) have shown that many strains of R pneumococci can be reverted to the S form by repeated mouse passages or by cultivation *in vitro* in anti-R serum. Griffith (12) has shown that R pneumococci frequently revert when they are mixed with large doses of heat-killed S pneumococci and inoculated subcutaneously into mice.

We have attempted to revert glossy hemolytic streptococci to the matt form by each of these three methods.

1. Mouse Passage.—Passage experiments have been done with glossy cultures derived from five different strains (S3, S23, Henson, S43, C203) but no definite evidence has been obtained that reversion can be achieved by this method. In two cases, S3 (ten passages) and Henson (twenty-five passages), the glossy character of the cultures was judged entirely by the appearance of the colonies as no anti-M serum was available for these strains.

The virulence (0.1 cc.) and colony form of Strain S3 remained unchanged after passage through ten mice.

Passage of Strain Henson through twenty-five mice caused the virulence of the culture to rise from 0.5 cc. to 0.01 cc. and this change was accompanied by a slight alteration in colony form, many of the colonies in the passage culture having flat tops instead of the typical dome-shaped appearance of glossy colonies. In this instance the partial restoration of virulence and the accompanying change in colony structure may possibly indicate that reversion had commenced but the evidence is inconclusive in the absence of serological proof that the culture had been completely degraded before the mouse passages were commenced.

The glossy culture of Strain S23 appeared to be completely degraded as no trace of precipitate was formed when highly concentrated HCl extracts were mixed with the homologous type-specific antibody. This culture was passed through twenty-seven mice and at the end of the experiment the virulence for mice (0.01 cc.) and the colony form remained unchanged and there was no reappearance of type-specific substance.

The glossy culture of Strain S43 was not completely degraded as, although unconcentrated extracts of the variant culture did not precipitate the homologous anti-M serum, yet traces of type-specific substance could be demonstrated in concentrated HCl extracts. This culture was passed through a series of mice and examination of unconcentrated extracts, after each passage, showed the gradual reappearance of type-specific substance so that after eight passages the culture was equal to the original matt form in its yield of type-specific substance. The virulence (0.1 cc.) and colony form were unaltered by eight passages but this experiment seems to indicate that a culture which has lost the major part of its type-specific substance and yet retained a fraction of its original specificity can be reverted to the original form with comparative ease.

The variant culture of Strain C203 formed typical glossy colonies but concentrated HCl extracts of the cocci contained traces of type-specific substance. After passage through ten mice the virulence (0.1 cc.) and the colony form of the culture remained unchanged and there was no increase in the quantity of type-specific substance which could be extracted from the cocci.

2. Cultivation in Immune Serum.—A glossy culture of Strain New York V was obtained which failed to kill mice in a dose of 0.5 cc. Rabbits were immunized with this culture and a serum was obtained which agglutinated the glossy culture up to a dilution of 1 in 2,560. The glossy culture was grown in various dilutions of this serum (5 per cent, 10 per cent, 50 per cent, 100 per cent) for a number of transfers but in no case was there any evidence of reversion—50 per cent serum was selected as the concentration in which the cocci appeared to multiply most freely and the culture was carried 118 transfers in this medium. At the end of the experiment the form of the colonies and the virulence of the culture remained unchanged.

Immune serum prepared against glossy cocci contains antibodies to the two non-type-specific fractions, P and C. In the following experiment the influence

of pure anti-P serum on glossy cultures of four strains was tested. High titer anti-P serum was prepared by immunizing rabbits with purified nucleoprotein extracted from hemolytic streptococci. The glossy variant forms of four strains (S43, S23, C203, London) were cultivated for twelve transfers in a 10 per cent dilution of this serum. This treatment did not alter the colony forms of the cultures although the virulence of two strains (S23 and London) was definitely increased. The quantities of type-specific substance, however, which could be extracted from the cocci of all four strains remained unchanged.

3. Subcutaneous Inoculation of Mice with Glossy Cultures in Combination with the Homologous Matt Cocci Killed by Heat.—A few experiments have been done with one of our glossy strains (Henson) in an attempt to revert this culture to the matt form by the technique devised by Griffith for the reversion of R pneumococci to the S form. A heavy suspension of the matt culture was prepared by heating, at 60°C. for 30 minutes, the deposit from 50 cc. of culture, concentrated to 2 cc. 0.5 cc. of the heat-killed suspension was mixed with 0.05 cc. of living glossy culture and injected subcutaneously into a mouse. Cultures from the lesion in the mouse contained both matt and glossy colonies although controls indicated that the matt organisms of the heated suspension were dead. Unfortunately, this technique, so successful with pneumococci, is not altogether ·satisfactory for hemolytic streptococci as it has been found impossible to avoid ulceration when large numbers of heat-killed matt organisms are combined with glossy cultures and even small doses of glossy culture alone frequently cause ulceration. Matt colonies isolated from these open ulcers must be viewed with suspicion since they may arise from contaminating cocci, but it seems probable that further work with this technique may yield convincing evidence of the reversion of the glossy cocci to the matt form.

So far as any conclusions can be drawn from the limited number of observations recorded it seems that the glossy variant is a highly stable form but that reversion may occur under certain conditions.

A Comparison of the Toxigenicity of Matt and Glossy Cultures of the Same Strain.

The method used for comparing the toxigenicity, the virulence for mice and the colony appearance of matt and glossy cultures of the same strain was as follows:

Young broth cultures of the different forms of each strain were sown in 50 cc. of tryptic digest broth. After 16 hours incubation the virulence and colony appearance of a sample taken from each culture were determined; and the flasks were then returned to the incubator. The cultures were filtered after 4 days of incubation and the filtrates were tested by injecting 0.1 cc. of diluted filtrate into

TABLE III.

A Comparison of the Toxigenicity and Virulence of Matt and Glossy Cultures of the Same Strain.

	Identification number of patient	Filtrate from matt virulent culture	Filtrate from matt attenuated culture	Filtrate from glossy culture
1. Bronchopneumonia strains				
Strain S43. Active filtrate diluted 1 in 500 " " Boiled " " 1 " 500	1	15 mm. (10⁻⁷) 11 "	15 mm. (10⁻¹) 10 "	13 mm. (10⁻¹) 8 "
" " Active " 1 " 50 " " Boiled " 1 " 50	1	27 " 18 "	23 " 9 "	26 " 14 "
" S23. Active " 1 " 100 " " Boiled " 1 " 100	1	22 " (10⁻⁷) 6	— —	20 " (10⁻²) 11 "
2. Scarlet fever strains				
Strain C203. Active filtrate diluted 1 in 1,000 " " Boiled " 1 " 1,000	1	28 " (10⁻⁷) 2 "	— —	20 " (less than 10⁻¹) 7 "
N. Y. V. Active " 1 " 1,000 " " Boiled " 1 " 1,000	2	18 " (10⁻⁵) 0	— —	19 " (10⁻¹) 0
" " Active " 1 " 1,000 " " Boiled " 1 " 1,000	3	26 mm. (10⁻⁵) 0	— —	32 mm. (10⁻¹) 0
" " Active " 1 " 1,000 " " Boiled " 1 " 1,000	4	10 mm. (10⁻⁵) 0	— —	10 mm. (10⁻¹) 0
" " Active " 1 " 1,000 " " Boiled " 1 " 1,000	5	11 mm. (10⁻⁵) 0	— —	18 mm. (10⁻¹) 0

The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared. 0 indicates no reaction; — indicates not tested.

the skin of one, or more, known positive reactors. Table III gives the dimensions of the reactions which followed the injection of active filtrate and of the same filtrate after heating in boiling water for 2 hours. Measurements, which are given in mm. representing the average diameter of the skin reactions, were taken 24 hours after injection. The figures in brackets give the virulence for mice of the cultures from which the filtrates were prepared.

The two strains isolated from cases of bronchopneumonia produced weak toxic filtrates in comparison with the scarlet fever strains and were therefore used in greater concentration.

It will be seen from Table III that the filtrates from the different forms of each strain caused approximately equal reactions and that no correlation could be established between virulence and toxigenicity.

DISCUSSION.

It may be stated as a broad generalization that the type-specific substance M is present in the potentially virulent organisms comprising the matt variety of colony and that it is not present in the avirulent variant cocci which form glossy colonies. This is analogous to the invariable presence of the soluble specific substance S in virulent cultures of pneumococci and its absence from avirulent R cultures, but here the analogy breaks down as far as virulence for mice is concerned, since it is possible to prepare matt cultures of hemolytic streptococci which contain large quantities of the type-specific substance M and yet are avirulent for mice.

One of the most striking characteristics of hemolytic streptococci is the difficulty which has always been experienced in securing highly virulent cultures of a large number of strains. This is undoubtedly due in part to the fact that the glossy variant is a highly stable avirulent form but even when we exclude this variant form and confine our attention to the matt or potentially virulent varieties we are still unable to secure highly virulent cultures with any degree of ease. The behavior of matt attenuated cultures undergoing mouse passage is frequently capricious; virulence generally rises to a moderate level after the initial ten, or twenty passages and it may then increase suddenly, or it may gradually increase after many more passages, or it may remain for an indefinite period in a state of mediocrity. This is in contrast to pneumococci which appear to be either rough and avirulent for mice or smooth and of maximal virulence for this

species. The virulence of pneumococci appears to be intimately associated with the presence or absence of the soluble specific substance S; in the case of hemolytic streptococci, however, virulence is not entirely dependent on the presence or absence of the type-specific substance M; some additional unknown factor is operative. Glossy variants, when fully degraded, contain no type-specific substance and are avirulent for mice; matt organisms occur in two forms equally rich in type-specific substance—one of these forms is no more virulent for mice than the glossy variant, the other is highly virulent.

It is possible that this contrast between pneumococci and hemolytic streptococci may be partly due to differences in bacterial structure. In the case of pneumococci the soluble specific substance S is disposed in a capsular layer over the surface of the organism; but microscopic examination of hemolytic streptococci gives no information as to the situation of the type-specific substance M in the bacterial bodies; and it is possible that the distribution of this substance throughout the organisms may render it less accessible and therefore less susceptible to external influences.

Certain strains of hemolytic streptococci exhibit an unexpected stability in all forms. Cultures which are partially degraded so that they contain only small quantities of type-specific substance may be passed through a number of mice without any accumulation of type-specific substance and without any alteration of virulence. Conversely, when the virulence of a matt strain has become established it is difficult to reduce the culture to the matt attenuated state and at the same time to avoid conversion to the glossy variant form.

No relationship could be established between toxigenicity and virulence; in some instances highly virulent matt cultures produced weak toxic filtrates and the glossy variant avirulent forms were equally toxigenic; in other instances relatively avirulent matt strains produced highly toxic filtrates.

It appears therefore that virulence is not determined by toxigenicity and is not entirely depend ant on the presence or absence of type-specific substance although cultures which have lost their type specificity are invariably avirulent.

An unknown factor determines whether hemolytic streptococci,

which contain their full quota of type-specific substance, are virulent or attenuated.

SUMMARY.

Hemolytic streptococci, when freshly isolated from pathogenic lesions, form characteristic matt colonies and contain the type-specific substance M.

Two varieties of matt cultures, equally rich in type-specific substance, can be distinguished by the virulence of the organisms for mice: (1) the matt virulent variety, (2) the matt attenuated variety.

The matt forms of hemolytic streptococci can be degraded to a third variety which forms glossy colonies and is always relatively avirulent. This is accomplished by prolonged cultivation on artificial media, by selection of colonies or by cultivation in homologous anti-M serum. In the process of degradation the cocci lose the major part of their type-specific substance but complete disappearance of type-specific substance rarely occurs.

The glossy variant form, when fully degraded, is highly stable; but glossy cultures which have retained some type-specific substance can occasionally be reverted to the original matt form.

Toxic filtrates from matt and glossy cultures are approximately equal in skin reactivity.

No relationship appears to exist between virulence and toxigenicity.

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ANTIGENIC DIFFERENCES BETWEEN MATT HEMOLYTIC STREPTOCOCCI AND THEIR GLOSSY VARIANTS.

By R. C. LANCEFIELD, Ph.D., and E. W. TODD,[*] M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

In a previous communication (1) it was shown that hemolytic streptococci in the matt form contain the type-specific substance M and that when matt cultures are degraded to the glossy variant form the organisms lose their type-specific substance. The antigenic differences between matt and glossy cultures, which are described in the present paper, were demonstrated with four different strains of hemolytic streptococci.

Owing to individual differences between the four strains it is essential to describe each series of experiments separately.

A. Strain S43.

This strain was isolated from a case of bronchopneumonia and had been kept in stock cultures for 10 years before our work was commenced. In spite of this long interval, however, the culture was entirely composed of matt colonies. Virulence tests proved that 0.1 cc. killed mice regularly in 24 hours but mice receiving 0.01 cc. survived indefinitely.[1]

The culture was, therefore, in the matt attenuated state and efforts were made to obtain the matt virulent form and the glossy variant so

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[*] British Medical Research Council Fellow.

[1] A standard technique was used in all the virulence tests recorded in this paper. Cultures were sown in tryptic digest broth and incubated for 16 hours. Tenfold dilutions of culture, in infusion broth, were injected intraperitoneally into a series of white mice, each mouse receiving 0.5 cc. of the appropriate dilution. In some cases counts were made by plating dilutions in blood agar and although this was not done as a routine in every test, a sufficient number of observations were made to show that the dose representing 0.000001 cc. of culture usually contained about 100 organisms.

that the three forms of the same strain could be compared and their antigenic relationships established.

The matt virulent form was secured by passing the original culture through a series of mice (0.1 cc. or 0.2 cc. of peritoneal washings being transferred directly from mouse to mouse), and, although there was an initial increase of virulence, after the first few passages, it required 51 passages before 0.000001 cc. of culture could be relied upon to kill mice regularly.

It was even more difficult to obtain the glossy variant from this highly stable matt strain, as prolonged subcultivation on agar slants did not alter the typical

TABLE I.

Precipitin Reactions of Extracts of S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy with the Homologous Pure Anti-M Serum from Which Non-Type-Specific Antibodies Had Been Removed by Absorption with a Heterologous Strain.

Anti-M serum absorbed with heterologous strain*	Volume of extracts†	Precipitates with extracts from		
		S43 matt virulent	S43 matt attenuated	S43 glossy
0.2 cc.	0.3 cc.	+++	+++	−
0.2 cc.	0.1 cc.	++	++	−
0.2 cc.	0.025 cc.	+	+	−

* Serum R166, against Strain S43 matt attenuated.

† These volumes of crude HCl extract were made up to 0.3 cc. with saline. The tests were incubated 2 hours at 37°C. and read after standing in the ice box overnight.

+++, ++, + represent degrees of precipitate.

− represents no precipitate.

matt appearance of the colonies. The original culture was, therefore, grown in undiluted, high titer anti-M serum, prepared by immunizing rabbits with the matt attenuated form, and after sixteen transfers in this medium the appearance of the colonies seemed to indicate that the culture had been reduced to the glossy state.[2] Table I shows the precipitin reactions of HCl extracts prepared from the three forms of culture with pure anti-M serum previously absorbed with a heterologous strain to remove antibodies to P and C.

Extracts of the matt virulent and of the matt attenuated cultures formed equally heavy precipitates with anti-M serum but a similar extract of the glossy

[2] Strain S43 in the matt state formed typical matt colonies, but colonies of the variant culture were characterized by a heaped up center surrounded by a wide flat margin and they never assumed the typical glossy appearance.

culture did not cause any precipitation.[3] This experiment showed that the variant contained little, if any, type-specific substance but subsequent examination of a concentrated extract proved that the culture was not entirely free from the type-specific fraction. An HCl extract was made from the deposit of 9 liters of culture, it was then concentrated by precipitation with alcohol and redissolved in 5 cc. of saline. This concentrated and purified extract caused some precipitation with the pure anti-M serum showing that the culture still retained traces of type-specific substance. Further attempts to reduce this strain to a completely degraded state were unsuccessful; colony selection was combined with more than 120 transfers in immune serum but even after this treatment traces of type-specific substance could still be detected.

Three kinds of antiserum were prepared by immunizing three groups of rabbits with the three forms of Strain S43. Four rabbits were included in each group and all received equal doses of the appropriate cultures on the same days—the whole series being finally bled on the same day. The immune sera were used for precipitin reactions and for mouse protection tests; in the latter tests, 0.5 cc. of serum was injected into the peritoneal cavity of a series of mice and the degree of passive immunity conferred was tested, on the following day, by inoculating the mice intraperitoneally with graduated doses of virulent culture.

Precipitin Tests.—Table II gives the results of precipitin reactions with the three kinds of sera and purified solutions of the nucleoprotein P, the carbohydrate C and the homologous type-specific substance M.

The non-type-specific fractions P and C precipitated the three kinds of sera to a similar extent though, as usual, the anti-glossy sera contained, in the aggregate, an excess of C antibody. The type-specific substance M, in contrast to the P and C fractions, formed heavy flocculent precipitates with the anti-matt sera but only traces of precipitate with the anti-glossy sera. The appearance of these traces of precipitate confirmed the observation, already noted above, that the S43 glossy form was not completely degraded by showing that it

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[3] Crude HCl extracts of glossy cultures when fully degraded, are free from type-specific substance but they contain the non-type-specific fractions P and C and will consequently precipitate *unabsorbed* antibacterial serum prepared against any strain of hemolytic streptococcus. Precipitates due to the C fraction are easily recognized as they appear late and form compact discs quite unlike the flocculent precipitates of the type-specific fraction which begin to appear as soon as the extract comes in contact with the serum. Precipitates due to the nucleoprotein P are less flocculent than those of the type-specific fraction M and appear as a diffuse cloud; the use of absorbed serum is, however, necessary to distinguish between these two forms of precipitate.

TABLE II.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain S43 (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

Volume of extracts and concentration of P*	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
Purified solution of homologous type-specific substance M from S43 0.4 cc.	++	++	+++	++	++	++++	++±	±	±	±	+
0.1 cc.	++±	++	+++±	+++±	+++±	++++	++	±	+	±	+
0.025 cc.	++±	+	++	+	++	+	++±	±	+	±	+
Purified solution of species-specific carbohydrate C 0.4 cc.	+++	+++	±	+++	+	++±	+	++±	+++	+++	++±
0.1 cc.	++	++	++	+++	++	+++	++	++	+++	+++	++
0.025 cc.	+	++±	++	++±	++±	++±	±	++±	+±	+±	+
Purified solution of nucleoprotein P 1 in 1,000	+	+	+	+	+	+	±	+	+	+	+
1 in 4,000	+	+	+	+	+	+	-	+	+	+	±
1 in 16,000	-	±	±	±	±	+	-	-	-	±	±

* These volumes were made up to 0.4 cc. with saline, and 0.1 cc. of serum was added to each tube.

produced traces of type-specific antibody when used as an antigen for immunizing rabbits.

TABLE III a.

Passive Protection of Mice against S43 Matt Virulent by Three Kinds of Sera, Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture				Sera prepared with matt attenuated culture			Sera prepared with glossy culture			
		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
0.00001 cc.	†72	S	S	See Table III b	S	S	See Table III b	S	S	S	†24	S
0.0001 cc.	†24	S	S		S	S		†77	†90	S	†21	†27
0.001 cc.	†21	S	S		S	†21		S	S	†22	†21	S
0.01 cc.	—	S	S		†22	†21		S	†21	†21	†21	†21
0.1 cc.	—	†21	†29		†24	†21		S	†21	†21	†21	†21

In all tables the following symbols are used.

S indicates that the mice survived for 8 days and were then discarded.

† indicates death of mice.

Numerals indicate number of hours between time of injection and finding mice dead.

— indicates that the test was not done.

The mice received 0.5 cc. of serum intraperitoneally and 24 hours later test doses of virulent culture were injected into the peritoneal cavity.

TABLE III b.

Passive Protection of Mice against S60 Matt Virulent (Same Type as S43) by Three Kinds of Serum Prepared against S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent culture	Control normal mice	Serum prepared with S43 matt virulent culture A3	Serum prepared with S43 matt attenuated culture A6	Serum prepared with S43 glossy culture A11
0.000001 cc.	†45	S	S	S
0.00001 cc.	S	†45	S	†69
0.0001 cc.	†45	S	S	†45
0.001 cc.	†21	S	S	†23
0.01 cc.	†21	S	S	†21
0.1 cc.	†21	†21	†21	S

Passive Protection of Mice.—Table III a shows the degree of passive immunity conferred on mice by these sera against a virulent culture

of the homologous strain and Table III *b* shows the protection against a virulent culture of S60, a strain identified by Dochez, Avery and Lancefield (2) as belonging to the same type as S43.[4]

TABLE IV *a*.

Active Immunization of Mice against S43 Matt Virulent by Vaccines Prepared with S43 Matt Virulent, S43 Matt Attenuated and S43 Glossy.

Test doses of virulent S43 culture	Control normal mice	Mice previously inoculated with S43 vaccines*			Controls Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with matt attenuated culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	†31	S	S	†24	†28	†71
0.00001 cc.	†28	S	†29	†23	†23	†27
0.0001 cc.	†29	S	S	†23	†23	†23
0.001 cc.	†23	†80	†23	†23	†23	†23
0.01 cc.	†23	†23	†23	†23	†23	†23
0.1 cc.	†23	S	†47	†23	—	†23

* Vaccines = undiluted 16 hour broth cultures heated at 56°C. for 1 hour.

TABLE IV *b*.

Showing that the Actively Immune Mice Surviving the Test Recorded in Table IV a Were Not Protected against a Matt Virulent Culture of a Heterologous Strain S23.

Test doses of virulent S23 culture	Control normal mice	Mice protected with S43 matt vaccines which had survived test doses of S43 matt virulent
0.000001 cc.	S	†23
0.00001 cc.	†23	†23
0.0001 cc.	†144	†23
0.001 cc.	†23	†23
0.01 cc.	†23	†23
0.1 cc.	†8	†23

The sera prepared against the matt virulent and matt attenuated cultures afforded an equal amount of protection to mice and the

[4] Most of the protection tests recorded in this paper were only done once and no attempt has been made to correct obvious discrepancies due to the natural resistance of individual mice by repeating the tests.

anti-glossy sera also showed some protective power. The small degree of protection conferred on mice by the anti-glossy sera corresponds to the small quantity of type-specific antibody contained in the sera (see Table II) and this coincidence becomes significant when we observe, from the study of strain S23 that serum, prepared with completely degraded glossy cultures which are devoid of type-specific substance, does not contain any type-specific antibody and does not protect mice.

Active Immunization of Mice.—Table IV records the results obtained when mice, actively immunized with the matt virulent, matt attenuated and glossy cultures, were tested for immunity.

Tryptic digest broth cultures of the three forms of Strain S43 were incubated for 16 hours and then killed by heating at 60°C. for an hour. These heat-killed cultures, undiluted and suspended in the broth in which they had grown, were inoculated intraperitoneally into three series of mice. During the 1st week the mice received 0.1 cc. doses on 4 successive days, during the 2nd week four 0.2 cc. doses and during the 3rd week four 0.4 cc. doses. 10 days after the final inoculation the active immunity of the mice was tested by intraperitoneal injection of graduated doses of the matt virulent culture. The controls for this experiment were (1) a series of normal untreated mice, (2) a series of mice inoculated with corresponding doses of tryptic digest broth, (3) a series of mice treated with the matt virulent vaccine of a heterologous strain, S23.

The mice vaccinated with the matt cultures in both the virulent and attenuated forms showed evidence of protection; but mice vaccinated with the glossy form were not protected against infection with the matt virulent cocci.

The specificity of active immunization is also demonstrated by this experiment. Vaccination with a heterologous matt virulent Strain S23 did not afford any protection against Strain S43 and Table IV *b* shows that there was no cross-immunity when protected mice surviving from the first experiment and immune to S43 were tested 10 days later for immunity to S23.

B. Strain S23.

This strain, isolated from a case of bronchopneumonia at the same time as Strain S43, had been used by Andrewes, Derick and Swift (3) as the test organism for protection experiments, and in the course

of their work its virulence had been increased by passage through a number of mice.

Virulence tests showed that 0.000001 cc. of culture killed mice regularly and occasionally 0.0000001 cc. and even 0.00000001 cc. proved a fatal dose.

The colonies had an undulating contour which made observations on the light-reflecting properties of their surfaces difficult and this peculiarity persisted when the strain was converted to the glossy state. Cultures of the virulent form usually contained many pseudoglossy colonies and, although a large number of plate cultures were examined, it was only rarely that either matt or glossy colonies appeared in typical forms.

Owing to these peculiarities we relied entirely on precipitin tests, by which the quantity of type-specific substance in a culture can be assessed, to judge when this strain was completely degraded to the variant form. The matt virulent strain was cultivated in homologous high titer anti-M serum and after 55 transfers in 50 per cent serum, concentrated extracts of the culture did not precipitate the homologous anti-M serum; and a single mouse passage did not cause the reappearance of type-specific substance. This variant culture, which, measured by the absence of type-specific substance, was completely degraded, was nevertheless partially virulent, 0.01 cc. being the M.L.D. for mice.

It was difficult to obtain a matt attenuated culture as the peculiarities of the strain made selection of colonies impossible. After nine transfers in homologous immune serum, containing only a small amount of type-specific antibody, the virulence of the culture was reduced from 0.000001 cc. to 0.001 cc. although the quantity of type-specific substance, measured by precipitin reactions, remained unchanged. This culture probably represented the matt attenuated form of Strain S23 as it was only slightly more virulent than the glossy variant; but owing to its high virulence, compared with the matt attenuated cultures of other strains, it was not used in our experiments.

Precipitin Tests.—Two series of rabbits were immunized with the matt virulent and glossy forms of Strain S23; Table V shows the precipitin reactions of their sera. It will be seen that all the sera

TABLE V.

Precipitin Tests on Two Kinds of Sera, Prepared against S23 Matt Virulent and S23 Glossy, with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
	B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
Purified solution of homologous type-specific substance M from S23										
0.4 cc.	++	++	++	±	+++	−	−	−	−	−
0.1 cc.	+++±	+++±	+++±	±	+++	−	−	−	−	+±
0.025 cc.	+±	++	+±	+	+	−	−	−	−	++
Purified solution of species-specific carbohydrate C										
0.1 cc.	−	+±	+±	−	−	+	±	−	±	+
0.1 cc.	−	+±	++	−	−	++	++	++	+	+±
0.025 cc.	−	+++±	+++±	−	−	++±	++	++	+	++
Purified solution of nucleoprotein P										
1 in 1,000	±	±	±	−	±	−	±	+±	±	±
1 in 4,000	−	−	−	−	±	−	−	+±	−	−
1 in 16,000	−	−	−	−	−	−	−	±	−	−

prepared against the matt virulent culture were precipitated by an extract containing the homologous type-specific substance but the sera prepared against the glossy variant remained perfectly clear

TABLE VI.

Absorption of Anti-Matt and Anti-Glossy Sera with the Homologous Matt and Glossy Cultures. Strain S23.

	Volumes of extracts	Serum B6 prepared with matt virulent culture of Strain S23			Serum B9 prepared with glossy culture of Strain S23		
		Control unab- sorbed	Ab- sorbed with S23 matt virulent	Absorbed with S23 glossy	Control unab- sorbed	Ab- sorbed with S23 matt virulent	Ab- sorbed with S23 glossy
Purified solution of homolo- gous type-specific sub- stance M from S23	0.4　cc.	++±	−	++±	−	−	−
	0.1　cc.	++±	−	++±	−	−	−
	0.025 cc.	+	−	+	−	−	−
Purified solution of species- specific carbohydrate C	0.4　cc.	−	−	−	−	−	−
	0.1　cc.	−	−	−	+±	−	−
	0.025 cc.	−	−	−	++	−	−

TABLE VII.

Passive Protection of Mice against S23 Matt Virulent by Two Kinds of Serum Prepared against S23 Matt Virulent and S23 Glossy.

Test doses of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with glossy culture				
		B1	B3	B4	B5	B6	B7	B8	B9	B10	B12
0.0000001 cc.	†67	−	−	S	−	S	†30	†21	†31	†23	†29
0.000001　cc.	†23	S	S	S	S	S	†21	†24	†25	†20	†29
0.00001　cc.	†23	†23	†23	S	†23	S	†21	†21	†26	†20	†23
0.0001　cc.	†23	S	S	S	†26	S	†21	†21	†23	†20	†23
0.001　cc.	−	†28	S	†96	S	S	†21	†21	†23	†20	†23
0.01　cc.	−	†21	†21	†27	†21	†72	†21	†21	†23	†20	†23
0.1　cc.	−	†21	†21	†21	†21	S	†21	†21	−	†20	†23

when mixed with the same extract. Both kinds of immune sera were precipitated by the non-type-specific fractions P and C, the anti-glossy sera being as usual more uniformly rich in antibody to the carbohydrate fraction C.

The anti-matt serum (B6) and the anti-glossy serum (B9) were chosen from the two groups of sera for absorption experiments. Both sera were absorbed with homologous matt virulent cocci and also with homologous glossy cocci. Table VI shows the result of this experiment.

The type-specific antibody was completely removed from the anti-matt serum by absorption with the homologous matt culture but it was unaffected by absorption with the homologous glossy culture.

The anti-glossy serum contained no type-specific antibody but its non-type-specific antibody was completely removed by absorption with both forms of the homologous strain.

TABLE VIII.

Active Immunization of Mice against S23 Matt Virulent by Vaccines Prepared with S23 Matt Virulent and S23 Glossy.

Test dose of virulent S23 culture	Control normal mice	Mice previously inoculated with S23 vaccines		Controls Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared with matt virulent culture	Vaccine prepared with glossy culture	Vaccine prepared with heterologous matt virulent culture	Inoculated with broth
0.000001 cc.	S	S	†24	†41	†41
0.00001 cc.	†63	†41	†21	†41	†26
0.0001 cc.	†23	†41	†22	†22	†21
0.001 cc.	†21	†110	†21	†21	†41
0.01 cc.	†21	†17	†17	†17	†17
0.1 cc.	†17	†13	†17	†22	†17

Passive Protection of Mice.—The sera prepared against the two forms of Strain S23 were used for mouse protection experiments with the homologous matt virulent culture as the test organism.

All the sera prepared against the matt virulent form afforded some degree of protection to mice but the anti-glossy sera did not confer any protection.

Active Immunization of Mice.—Two series of mice were inoculated with vaccines prepared from the matt and glossy forms of Strain S23; the dosage and technique were the same as those used in a similar experiment, previously recorded, with Strain S43. Controls con-

sisted of a series of normal mice, a series of mice inoculated with a heterologous matt virulent vaccine and a series of mice inoculated with broth (Table VIII).

In this experiment vaccines prepared from both forms of Strain S23 failed to confer immunity on the mice.

C. Strain C203.

This strain, from the collection of the Laboratories of the New York State Department of Health, was originally isolated from a

TABLE IX.

Precipitin Tests on Two Kinds of Sera, Prepared against C203 Matt Virulent and C203 Glossy with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture			Sera prepared with glossy culture			
		C1	C2	C3	C4	C5	C6	C7
Purified solution of homologous type-specific substance M from C203	0.4 cc.	++±	++	++	±	±	−	±
	0.1 cc.	++	+±	+	−	−	±	−
	0.025 cc.	+	+	±	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	−	−	−	−	+	−	+±
	0.1 cc.	−	−	−	++	+++±	−	+++
	0.025 cc.	±	−	−	++±	++	−	+++±
Purified solution of nucleoprotein P	1 in 1,000	+±	±	+	++	++±	+	+++±
	1 in 4,000	+	±	±	+±	+	±	+±
	1 in 16,000	−	−	−	±	±	−	±

case of scarlet fever and had been kept in stock culture for a considerable time before our experiments commenced. It was found to be entirely composed of typical matt colonies; and virulence tests showed that the M.L.D. for mice was 0.0000001 cc. or 0.00000001 cc.

The glossy variant form of this strain was obtained by selection of colonies combined with cultivation in 50 per cent homologous anti-M serum of high titer. After 27 transfers the virulence for mice had fallen from 0.0000001 cc. to 0.1 cc. and the culture formed typical glossy colonies; but traces of type-specific substance could still be

detected in unconcentrated HCl extracts. Continued efforts to secure a completely degraded culture did not cause any further decrease in the quantity of type-specific substance which the organisms contained.

Attempts to reduce this strain to the matt attenuated form were unsuccessful as any reduction in virulence was always accompanied by a partial loss of type-specific substance and by the appearance of atypical matt colonies which tended to assume the characteristics of glossy colonies.

Precipitin Tests.—Rabbits were immunized with the matt virulent and glossy forms of this strain; Table IX gives the precipitin reactions of the immune sera.

In this instance a departure was made from the technique used for immunizing rabbits with the other three strains; the organisms were washed to remove the toxin as the use of unwashed broth cultures of highly toxic strains involved the loss of a relatively large proportion of rabbits during immunization.

The results of precipitin tests were similar to those previously recorded in reference to other strains; the sera prepared with the matt virulent organisms gave good precipitates with the homologous type-specific substance M but only traces of precipitate with the non-type-specific fractions C and P; on the other hand sera prepared with glossy organisms gave only traces of precipitate with the type-specific substance M and comparatively good precipitates with the non-type-specific substances C and P.

The traces of type-specific antibody in the anti-glossy sera were a natural sequel of failure to secure this strain in the completely degraded form.

Passive Protection of Mice.—Table X*a* shows the results of protection tests with the different kinds of sera. All the anti-matt sera showed evidence of protective power and the anti-glossy sera with one exception also protected mice to some extent. This protection by sera prepared against the glossy form of Strain C203 is in harmony with the results of precipitin tests and with the original observation that the culture was not fully degraded but still contained type-specific substance. Table X*b* shows the specificity of protection by the anti-glossy sera. On reference to Table X*a* it will be seen that

TABLE X *a*.

Passive Protection of Mice against C203 Matt Virulent by Two Kinds of Sera Prepared against C203 Matt Virulent and C203 Glossy.

Test doses of virulent culture Strain C203	Control normal mice	Sera prepared with matt virulent culture			Sera prepared with glossy culture				Control mice inoculated with normal rabbit sera	
		C1	C2	C3	C4	C5	C6	C7	N1	N2
0.0000001 cc.	†24	S	S	S	†26	†25	S	S	S	†22
0.000001 cc.	†21	†24	S	†120	†22	S	S	S	†24	†28
0.00001 cc.	†21	S	S	S	†21	†25	S	†21	†21	†26
0.0001 cc.	†21	S	†168	S	†21	†25	†69	†21	†21	†21
0.001 cc.	†21	†24	†21	S	†21	S	†93	†21	†21	†21
0.01 cc.	—	†21	†21	†24	†21	†21	†21	†21	†21	†21

TABLE X *b*.

Specificity of Passive Protection of Mice by Anti-Glossy (C203) Serum.

Test doses of virulent culture Strain S23	Control normal mice	Serum prepared with glossy culture of Strain C203 C6
0.0000001 cc.	†53	†51
0.000001 cc.	†51	†22
0.00001 cc.	†22	†26
0.0001 cc.	†22	†56
0.001 cc.	†22	†22
0.01 cc.	—	†22

TABLE XI.

Active Immunization of Mice against C203 Matt Virulent by Vaccines Prepared with C203 Matt Virulent and C203 Glossy.

Test dose of virulent C203 culture	Control normal mice	Mice previously inoculated with C203 vaccines		Mice previously inoculated with heterologous matt virulent Vaccine S43 and with broth	
		Vaccine prepared from matt virulent culture	Vaccine prepared from glossy culture	Vaccine prepared from heterologous matt virulent culture	Inoculated with broth
0.0000001 cc.	†25	S	—	†40	†40
0.000001 cc.	†21	S	†24	†20	†25
0.00001 cc.	†22	S	†64	†16	†18
0.0001 cc.	†16	S	†20	†21	†16
0.001 cc.	†16	S	†16	†16	†16
0.01 cc.	†16	S	†17	†16	†16
0.1 cc.	†16	†20	†16	†16	†16

Serum C6 was probably the best anti-glossy serum for protection against the homologous matt virulent form of Strain C203; a series of mice was, therefore, injected with this serum and their immunity to a heterologous matt virulent Strain S23 was subsequently tested. Table X*b* shows that Serum C6 afforded no protection against the heterologous Strain S23.

Active Immunization of Mice.—Mice were vaccinated with the different forms of Strain C203 in the manner previously described; and their immunity was tested by inoculating graduated doses of a matt virulent culture of the same strain.

Table XI shows that vaccination with matt virulent organisms produced immunity against subsequent infection by the same strain; but vaccination with homologous glossy organisms, or with heterologous matt virulent organisms did not cause any immunity.

D. Strain London.

This strain, isolated by blood culture from a case of puerperal septicemia, had been kept in stock for about a year before our experiments began; and plate cultures showed that it was composed of a mixture of matt and glossy colonies. A pure matt culture, capable of killing mice in a dose of 0.01 cc., was obtained by selecting a suitable colony and the matt virulent form (M.L.D. 0.000001 cc.) was then prepared by passing this culture through 47 mice.

The matt attenuated culture, which was entirely composed of typical matt colonies indistinguishable from those of the virulent form and failed to kill mice when 0.5 cc. was injected intraperitoneally, was obtained by combining the selection of typical matt colonies with cultivation at 41°C.

The glossy variant was obtained by daily subcultivation of the original strain on agar slants without any selection of colonies or cultivation in immune serum. After 148 transfers on agar the culture was entirely composed of typical glossy colonies, and it was avirulent for mice (M.L.D. 1.0 cc.); but precipitin tests with concentrated HCl extracts of the organisms showed that this culture still retained traces of the type-specific substance M.

Precipitin Tests.—Table XII gives the precipitin reactions of the sera of three sets of rabbits immunized with the three forms of this

TABLE XII.

Precipitin Tests on Three Kinds of Sera, Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy) with Purified Solutions of Nucleoprotein P, Carbohydrate C and Homologous Type-Specific Substance M.

	Volume of extracts and concentration of P	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture					
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17
Purified solution of homologous type-specific substance M	0.4 cc.	++	++±	+++±	++++	+++	++±	+	++±	±	++	+	±	−	±	±	−	±
	0.1 cc.	++	++	++±	++±	+++	+	±	++±	±	++	+	−	−	−	−	−	−
	0.025 cc.	+	±	−	±	±	−	−	−	−	+	±	−	−	−	−	−	−
Purified solution of species-specific carbohydrate C	0.4 cc.	±	−	−	−	−	−	++	−	−	−	−	−	+±	−	++	++	−
	0.1 cc.	++	−	−	−	++	−	+++	−	−	++±	−	+±	++±	++	++++	+++±	+++±
	0.025 cc.	+++±	−	−	++±	+++	−	++	−	−	++	+	+++	+++±	+++±	++++	++++	++++
Purified solution of nucleoprotein P	1 in 1,000	++±	++	++±	++	+++	++	+	±	±	+	++	++	++	+	+	+	+
	1 in 4,000	++±	++	±	++	+++	++	+	±	−	−	+	+	±	±	±	±	±
	1 in 16,000	+	±	−	±	++±	−	±	−	−	−	−	−	−	±	−	−	−

TABLE XIII.

Passive Protection of Mice against Matt Virulent Form of Strain London by Three Kinds of Sera Prepared against the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of virulent culture	Control normal mice	Sera prepared with matt virulent culture					Sera prepared with matt attenuated culture						Sera prepared with glossy culture						Control mice inoculated with normal rabbit serum
		D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	
0.0000001 cc.	S	S	S	S	S	S	S	S	S	S	S	S	†69	†69	†45	†45	S	S	†30
0.000001 cc.	S	S	†45	†90	S	S	S	S	†90	S	S	S	†90	†45	†30	†21	†29	S	†76
0.00001 cc.	†21	S	S	†52	†45	S	S	†90	S	S	S	S	†55	†51	†31	S	†31	†55	†45
0.0001 cc.	†21	S	S	†31	S	S	S	†45	S	S	S	†90	†99	†45	†21	†31	†29	†30	†53
0.001 cc.	†21	S	†120	S	S	†140	S	†26	S	S	†160	S	†27	†21	†29	†21	†21	†21	†21
0.01 cc.	†21	S	†140	S	S	†26	†26	†26	S	S	†21	†21	†21	†21	†21	†21	†21	†21	†21

strain; and it will be seen that, as in previous similar experiments, the anti-matt sera were relatively rich in antibody to the type-specific substance M; while the anti-glossy sera had a preponderance of antibody to the non-type-specific fraction C.

Passive Protection of Mice.—Table XIII gives the results of protection tests with the sera prepared against the different forms of Strain London: both kinds of anti-matt sera protected mice against infection with the homologous matt virulent organisms but the anti-glossy sera, although they contained traces of type-specific antibody had little protective power.

TABLE XIV.

Active Immunization of Mice against the Matt Virulent Form of Strain London by Vaccines Prepared from the Three Forms of Strain London (Matt Virulent, Matt Attenuated and Glossy).

Test dose of matt virulent culture of Strain London	Control normal mice	Mice previously inoculated with vaccines prepared from Strain London in the following forms			Mice previously inoculated with heterologous vaccine prepared from S23 matt virulent and with broth	
		Matt virulent	Matt attenuated	Glossy	Heterologous matt virulent	Broth
0.0000001 cc.	†41	—	†89	—	†41	†41
0.000001 cc.	†27	†89	†41	†41	†25	†41
0.00001 cc.	†22	†70	†22	†24	†41	†27
0.0001 cc.	†22	S	†22	†22	†24	†17
0.001 cc.	†24	†65	S	†22	†22	†17
0.01 cc.	†17	†22	†17	†22	†17	†17
0.1 cc.	†17	†17	†17	—	†17	—

Active Immunization of Mice.—Table XIV shows the results of vaccinating mice with the three forms of Strain London and with matt virulent organisms of a heterologous strain. In this instance a final dose of 0.2 cc. of heat-killed culture was substituted for 0.4 cc. as used in other experiments because the mice began to lose weight during immunization. In this experiment, although there was little evidence of immunization by any of the vaccines, the mice vaccinated with the matt virulent organisms survived longer than any of the controls.

DISCUSSION.

Matt and glossy cultures of four strains of hemolytic streptococci, belonging to different serological types, were used to immunize rabbits. Only one of the four glossy cultures, Strain S23, was completely degraded to the point at which no trace of type-specific substance could be detected in highly concentrated HCl extracts of the organisms. The glossy cultures of the other three strains were not completely degraded as extracts from these organisms contained traces of the type-specific substance M.

Precipitin tests with the antisera of the four strains gave the following results: (1) All the anti-matt sera, whether prepared with virulent or attenuated organisms, contained antibody to the type-specific substance M. (2) The anti-glossy sera, prepared with cultures which were not fully degraded, were relatively deficient in type-specific antibody and gave either negative or weakly positive precipitin reactions with the type-specific substance M. (3) The five sera prepared with the completely degraded glossy form of Strain S23 did not contain any type-specific antibody. (4) The type-specific antibody was completely removed by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Experiments on passive immunization of mice showed that all the anti-matt sera had some protective power against infection with homologous matt virulent organisms. Some of the anti-glossy sera, prepared against strains which were not fully degraded, also protected mice but none of the anti-glossy sera prepared against the completely degraded glossy form of Strain S23 afforded any protection against infection with the homologous virulent organisms.

No exact parallel was established between the anti-M titer and the protective power of immune sera but high titer anti-M sera usually gave good protection.

It might be supposed that protection against infection with matt organisms by anti-matt sera and the absence of protection by anti-glossy sera merely represented specificity which could be demonstrated between different forms of the same strain and that anti-glossy sera would protect against infection with glossy organisms

better than anti-matt sera.   Three of the strains used in this investigation were unsuitable for experiments to test this possibility because the glossy organisms were completely avirulent for mice. The completely degraded glossy variant of Strain S23 was, however, relatively virulent for mice (M.L.D. 0.01 cc.) and comparison was, therefore, made between the protective power of anti-matt and of anti-glossy sera against infection with the homologous glossy organisms.   No evidence was obtained that either the anti-matt sera or the anti-glossy sera had any protective power against infection with glossy organisms.

There is considerable evidence that both passive protection and active immunity were antibacterial and not antitoxic; this evidence may be summarized as follows:

1. All the strains produced toxin to some extent but the protective action of the antisera was type-specific; sera prepared with toxigenic strains did not protect against infection with heterologous strains of equal toxigenicity.   The immunity due to vaccination was also type-specific.

2. The toxigenicity of the matt form and of the glossy form of Strain S23 was determined quantitatively by intracutaneous tests in human subjects, and it was found that the matt and the glossy forms were approximately equal in their power to produce skin-reactive toxin.   In spite of this equality, anti-glossy sera possessed no protective power against infection with homologous matt virulent organisms.

3. The anti-matt sera against Strain C203 were prepared with washed bacteria, and the production of antitoxin was, therefore, limited, but in spite of this limitation the sera protected against the highly toxigenic homologous strain but not against the weakly toxigenic heterologous Strain S23.

4. Active immunization of mice with whole broth cultures of the matt and of the glossy forms of the scarlet fever Strain C203 showed that, although the two forms were approximately equal in toxigenicity, the matt vaccine produced a high degree of immunity while the glossy vaccine produced no immunity against infection with homologous virulent organisms.

These observations, which show that the matt varieties of hemolytic

streptococci are type-specific while the glossy variant forms are not type-specific, are in agreement with the work of Andrewes. He also isolated two varieties of hemolytic streptococci corresponding to our matt and glossy forms, which, in a preliminary communication (4), he designates "rough" and "smooth" with the reservation "that they must not be supposed to correspond with the rough and smooth forms of *B. coli* and Salmonella." Using a special technique, he was able to establish by agglutination and absorption experiments that the "rough" forms exhibit considerable specificity; that the "smooth" forms of different strains are all alike serologically, and that the "rough" and "smooth" forms of a given strain are serologically distinct.

A comparison of his results with those recorded in this paper shows that his type-specific agglutination with hemolytic streptococci of the matt variety is in agreement with our observation that large quantities of the type-specific substance M are found in matt organisms; also his observation that anti-glossy serum agglutinates all strains of hemolytic streptococci, when in the glossy form, with complete impartiality is in agreement with our observation that anti-glossy sera contain more antibody to the non-type-specific fractions than anti-matt sera.

SUMMARY.

The matt and the glossy forms of four strains of hemolytic streptococci were used to immunize rabbits.

Precipitin tests showed that rabbit sera prepared against matt organisms, whether virulent or avirulent for mice, contained type-specific antibody while sera prepared against completely degraded glossy organisms contained no type-specific antibody.

Type-specific antibody was removed from the sera by absorption with homologous matt organisms but was unaffected by absorption with homologous glossy organisms.

Passive protection experiments on mice showed that anti-matt sera were protective and anti-glossy sera non-protective against infection with homologous virulent organisms.

Vaccination of mice with matt organisms rendered them immune to subsequent infection with homologous virulent cultures; but vaccination with glossy organisms established no active immunity.

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ACTIVE AND PASSIVE IMMUNITY TO PNEUMOCOCCUS INFECTION INDUCED IN RABBITS BY IMMUNIZATION WITH R PNEUMOCOCCI.

By WILLIAM S. TILLETT, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In a previous publication (1) the fact was established that rabbits, immunized with degraded, avirulent, non-type-specific pneumococci —so called R strains derived from any one of the three types—acquire a considerable degree of resistance against subsequent infection with virulent Type III pneumococci. Active resistance was demonstrable under these conditions in spite of the fact that the sera of the immunized rabbits contained no type-specific antibodies capable of agglutinating Type III S cells, or of precipitating the soluble specific substance derived from Type III cultures, or of conferring passive protection on mice against Type III infection. It was suggested (1) that this form of active immunity, effective in the absence of demonstrable type-specific antibodies and unrelated to the variety of pneumococcus used for immunization, differed in principle from type-specific immunity. The previous experiments were restricted to the study of the active resistance to Pneumococcus Type III. However, it seemed possible that this form of immunity—induced by immunization with R cells— might be effective against infection with other virulent types of pneumococcus. Consequently, additional studies have been carried on to determine whether the apparently non-type-specific resistance thus induced is as effective against Type I and Type II as against Type III. The results are recorded in this communication. In view of the differences which appear to exist between the form of active immunity stimulated in rabbits by type-specific S organisms and intimately associated with type-specific antibodies, and active resistance induced by prolonged immunization with R cells, further investigation into the nature and mechanism of the latter type of immunity is in progress.

In addition, the work has been extended to include passive immunity in order to determine whether the blood of rabbits immunized with R pneumococci, is capable of conferring protection upon normal animals of the same or of different species. The results of experiments on passive immunity are also included in this report.

Methods.

Antigens.—An R strain originally derived from Type II S culture (designated R_2), an R strain similarly derived from Type I S culture (designated R_1), and a Type III S strain were employed for the immunization of all rabbits used in the experiments on active immunity. Only the R_2 strain was used for immunization in the tests for passive immunity.

Vaccines were prepared from 12 to 14 hour plain broth cultures. The organisms, removed from the broth by centrifugation, were resuspended in physiological salt solution in such proportion that 0.5 cc. of the suspension was equivalent in bacterial content to 1 cc. of original culture, and heat-killed at 56° for 30 minutes.

Technique of Immunization.—The method of immunization, described by Cole and Moore (2), consisted of a total of 18 intravenous injections; 0.5 cc. doses of vaccine were given daily for 6 days followed by a week of rest until the procedure was repeated 3 times. As a rule, the animals were tested 9 to 14 days after the last dose.

Protection Tests.—12 to 14 hour blood broth cultures of virulent pneumococci of Types I, II, and III were used in all tests for active and passive immunity. Type I cultures possessed a maximum virulence for rabbits of 0.000001 cc.; Type II were usually fatal in 0.000001 cc. and always in 0.00001 cc. amounts; Type III killed regularly at 0.0001 cc. In most of the experiments, in order to follow the degree of the bacteremia, blood cultures were taken at frequent intervals during the course of infection as previously described (3).

Rabbits tested for increased resistance to Type I infection had previously been treated as follows: 1 had been immunized with R pneumococci derived from Type I S strain, 15 with R pneumococci derived from Type II S strain, and 3 with Type III S pneumococci. Their sera were tested for the presence of agglutinins for R organisms and for the S forms of the three specific types. In no instance were type-specific agglutinins demonstrable; there were, however, agglutinins for R cells present in titres of 1:640 to 1:1280. Antibody response was similar regardless of organisms used for immunization. That rabbit avirulent strains of Type III stimulate in rabbits antibody formation of the same character as that elicited by R cells has been previously described (4). The infecting dose of Type I pneumococcus, in every instance, represented 10,000 to 100,000 lethal doses. By keeping the test strain at a maximum virulence for rabbits of 0.000001 cc., 0.01 cc., or 0.1 cc. of such culture constituted the dosage of organisms employed.

In each experiment, in addition to the immunized rabbits, normal animals receiving the minimal lethal dose and others the test dose served as controls of the virulence of the culture.

TABLE I.

Active Immunity against Infection with Pneumococcus Type I in Rabbits Immunized with R Strains. (Three Rabbits Immunized with S_{III} Are Also Included.)

Number of rabbits	Immunized with	Infected with Pneumococcus Type III	Route of infection	Number died	Number survived
		cc.			
1	R_1	0.01	Intravenous	0	1
3	S_{III}	0.1	"	1*	0
		0.01	"	0	2
8	R_2	0.1	"	1†	5
		0.01	"	0	2
4	R_2	0.1	Intraperitoneal	1‡	0
		0.01	"	0	3
6	R_2	0.1	Intradermal	0	2
		0.01	"	0	4
Total 22...................................				3	19
9	Normal controls	0.000001	Intravenous	6	0
		0.000001	Intraperitoneal	1	0
		0.000001	Intradermal	2	0
Total.......................................				9	0

* Animal died 8 days after infection.

† Animal died 5 days after infection.

‡ Animal died 7 days after infection. Controls receiving test dose of culture died within 36 hours.

Active Immunity.

Rabbits were tested for resistance to infection by the injection of organisms intravenously, intraperitoneally, and intradermally (Table I). Blood cultures were taken at frequent intervals in order to observe the duration and degree of the bacteremia.

Rabbits Infected Intravenously.—Of 12 rabbits infected intravenously, 5 of which received 0.01 cc., and 7 0.1 cc. each of virulent Type I culture, 10 survived. The 2 which finally succumbed, lived 5 and 8 days respectively, whereas normal rabbits receiving the same dose died in 24 to 48 hours. As evidence of the duration of the immunity, 2 of the rabbits in this group were tested 4½ months after the last immunizing dose and found to be resistant. As demonstrated by blood cultures, organisms persisted in the circulation for 3 to 6 days, increasing and decreasing in number irregularly until their final disappearance. Even in the two fatal instances, the animals possessed some degree of partial immunity as revealed by the fact that both lived several days longer than the controls, that neither suffered an overwhelming septicemia, and that at autopsy each showed evidence of attempted localization in the form of pleurisy and pericarditis. A bacteremia characterized by an irregular course was previously shown to occur when R immunized rabbits were infected with a rabbit virulent strain of Type III pneumococcus (1). This form of bacteremia appears to be characteristic of the benign blood infection occurring when rabbits, immunized with R cells, are infected intravenously with any of the specific types of pneumococcus and suggests a similarity in the mechanism of recovery in each instance.

Rabbits Infected Intraperitoneally.—Four rabbits, immunized with R_2 organisms, were infected intraperitoneally; 1 received 0.1 cc. and 3 received 0.01 cc. of Type I pneumococci. The latter 3 animals survived; the one injected with 0.1 cc. lived 7 days. In the 3 animals which survived, only a few bacteria were transiently present in the blood stream. The duration of cocci in the peritoneal cavity was not determined.

Rabbits Infected Intradermally.—Type I organisms in doses of 0.01 cc. and 0.1 cc. were introduced intradermally into 6 rabbits which had previously been immunized with R_2 cells. All 6 animals survived. The local lesion developed rapidly; in 24 to 48 hours it appeared fulminating, usually reddish purple, edematous, spreading ventrally in a well defined and elevated band and forming a boggy pouch of edema over the more dependent portions of the abdominal wall. Areas of ecchymosis were commonly present. Normal rabbits reacted with a similar lesion, although succumbing to the infection. Furthermore, blood cultures revealed a striking difference in the course of the infection in normal and immunized animals. The blood stream of immune rabbits either remained sterile or contained only a few organisms transiently present. In sharp contrast, normal rabbits developed, within a few hours, a blood infection which increased rapidly in severity until death ensued from an overwhelming septicemia. Recently Goodner (5) has reported results obtained following intradermal injection of Type I pneumococci. The lesion which he describes as occurring in normal rabbits is identical with the inflammatory reaction encountered in the animals used in these experiments. Results which have been obtained following the intradermal inoculation of pneumococci into normal, type-specifically immune, and R immunized rabbits will be reported later in a separate communication.

In addition to the rabbits immunized with R organisms and tested for resistance to infection with Type I, 3 rabbits which had received similar preliminary injections were infected intravenously with virulent Type II pneumococci. They survived 10,000 lethal doses. The character of the blood infection and the process of recovery were similar in all respects to those already observed in the case of infection with Types I and III.

From the results of these experiments with Type I and Type II pneumococci, and from those reported (1) using Type III, it may be concluded that adequate immunization of rabbits with R pneumococci stimulates the development of active immunity which is effective against any of the fixed types. A consideration of these results and their possible significance will be presented in the discussion.

Passive Immunity.

In the course of an analysis of the immunity induced by repeated injections of R pneumococci, experiments have been carried out to determine whether this form of resistance is passively transferable. Whole citrated blood and serum of rabbits which have acquired resistance through immunization with R cells have been passively transferred to normal rabbits and also to mice. The R strain used for immunization was derived from Type II S culture. The infecting organisms were virulent S strains of Type I or Type III. These precautions were taken in order to minimize the possible participation of type-specific antibodies. The blood for transfusion was drawn 9 to 14 days after the last immunizing dose.

In the first experiments the procedure was to transfer whole blood or its constituents from resistant to normal rabbits and 24 hours later to inject the recipients with virulent pneumococci. An example of the results obtained following the passive transference of whole citrated blood, plasma, cells, and serum is given in Table II.

A description of this experiment will serve as an illustration of the method employed and the results obtained.

From the ear vein of an R immunized rabbit 20 cc. of blood was allowed to drop into a tube containing 0.5 cc. of a saturated solution of sodium citrate. This made in final dilution approximately a 2 per cent sodium citrate solution. Immediately upon obtaining the desired amount, the blood was injected by means of a syringe into the ear vein of a normal rabbit. In making transfusions from rabbit to

rabbit no precaution was taken with regard to blood grouping. Sometimes immediately after the operation the recipient would show evidence of shock, characterized by clonic and tonic muscular spasms. Complete recovery usually occurred in 3 to 4 minutes. In one instance death ensued and in 2 other animals permanent paralysis of the hind limbs resulted. 20 cc. of blood similarly collected in citrate were separated by centrifugation into plasma and cells. An equal amount without citrate was allowed to clot and the serum collected. Sterile

TABLE II.

Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood, Plasma, Cells, and Serum of Rabbits Immunized with R Pneumococci.

1. Protocol of Course of Bacteremia in Anti-R Donor and Recipients.

Time of blood culture	Number of colonies per unit of blood								Control 0.0001 cc. Type III
	Anti-R donor	Anti-R recipients				Normal donor	Normal recipients		
		Whole blood	Plasma	Serum	Cells		Whole blood	Serum	
2 hrs.	1	42	86	93	113	∞	20	1000	
6 hrs.	13	5	0	40	13	∞	2	816	
10 hrs.	1	9	15	62	142	∞	23	∞	
20 hrs.	58	15	7	12	518	D	148	∞	
24 hrs.	142	1	2	38	∞		272	D	
30 hrs.	32	0	29	116	∞		522	.	
48 hrs.	1	1	1	25	∞		∞		
72 hrs.	0	1	1	3	∞		D		D
96 hrs.	4	0	12	14	D				
5 days	0	0	1	8					
6 days	0	0	8	1					
9 days	0	0	0	0					
11 days	0[s]	0[s]	0[s]	0[s]					

0.1 cc. of rabbit virulent strain of Type III used as infecting dose.

D indicates death of the animal.

S indicates survival of the animal.

Numerals represent number of colonies per unit of blood.

precautions were observed throughout the procedure. 20 cc. of whole blood, or its equivalent in plasma, cells, or serum were then injected intravenously into normal rabbits. 24 hours later these rabbits were infected intravenously with 0.1 cc. of a rabbit virulent strain of Type III. Normal rabbits which had received comparable amounts of whole blood or serum from other normals were similarly infected. Other normal rabbits without preliminary treatment were infected with the maximal test dose and the minimal lethal amount of culture.

From Table II it may be seen that whole blood, plasma, and serum from resistant rabbits afforded protection against 1000 lethal doses of Type III pneumococcus, whereas blood cells alone were inadequate. Controls receiving normal blood or serum were unprotected. Tabulation of the number of organisms in the blood cultures reveals the fact that the resistant rabbits continued to have pneumococci in varying numbers in the blood stream from 3 to 6 days before permanent sterility was attained. Rabbits receiving normal blood, on the other hand, although possessing a slight initial capacity to reduce the number of circulating bacteria were unable to cope with the subsequent rapid increase, and died of an overwhelming septicemia.

Repetitions of protection experiments of this character using virulent Type I pneumococci instead of Type III gave results equally definite, indicating that, as in the case of active immunity, passive protection of rabbits is not limited as to type of infecting pneumococcus.

From these experiments it is established that resistance induced in rabbits by immunization with R pneumococci can be passively transferred to normal rabbits. In titering the amount of blood necessary to confer passive protection it was found that 15 to 20 cc. were necessary against doses of culture as high as those constantly employed, *i.e.* 1000 lethal doses of Type III or 10,000 to 100,000 of Type I. This quantity of blood was regularly used in all subsequent experiments. In Table III the total number of transfusions and the results are recorded. It may be seen that, with the amount of blood transfused and the dosage of culture kept constant, the time elapsing between transfusion and injection of organisms has been varied from 1 hour to 21 days. Of 5 rabbits infected within 5 hours of the time of transfusion 4 died and 1 survived. Twenty-three animals were infected 1 to 7 days after transfusion and of these 18 recovered. One rabbit infected 14 days after transfusion, survived, and of 5 in which an interval of 3 weeks elapsed, 3 recovered.

With the use of 8 to 10 cc. of serum, an amount comparable to that contained in 15 to 20 cc. of whole blood, protection was demonstrable but the results were somewhat less striking. In Table III the results of protection tests by the use of serum are presented. Of 5 rabbits infected 1 to 5 hours after serum administration, 4 survived. With an interval of 1 to 7 days, out of 9 animals tested, 4 survived; in 3 in-

TABLE III.

Passive Protection of Rabbits against Pneumococcus Infection by Transfusion of Blood and Serum of Rabbits Immunized with R Pneumococci.

2. Summary of Results of Passive Protection Tests in Rabbits.

Rabbit No.	Amount of blood	Time interval	Infection with			Result
	cc.					
1	20	1 hr.	0.1	cc. Type I		D 6 days
2	20	2 hrs.	0.1	" "	"	S
3	16	4 "	0.5	" "	III	D 4 days
4	15	4 "	0.5	" "	"	" 4 "
5	15	5 "	0.5	" "	"	" 4 "
6	20	24 "	0.1	" "	I	" 4 "
7	15	24 "	0.1	" "	"	S
8	20	24 "	0.5	" "	III	"
9	20	24 "	0.2	" "	"	"
10	20	24 "	0.2	" "	"	"
11	20	3 days	0.1	" "	I	"
12	20	3 "	0.1	" "	"	"
13	20	3 "	0.1	" "	"	D 4 days
14	20	3 "	0.01	" "	"	S
15	20	3 "	0.01	" "	"	"
16	20	3 "	0.5	" "	III	"
17	15	4 "	0.1	" "	I	D 4 days
18	15	4 "	0.1	" "	"	S
19	15	4 "	0.01	" "	"	"
20	20	7 "	0.01	" "	"	"
21	20	7 "	0.01	" "	"	"
22	20	7 "	0.1	" "	"	"
23	20	7 "	0.1	" "	"	D 4 days
24	17	7 "	0.1	" "	"	S
25	15	7 "	0.4	" "	III	"
26	15	7 "	0.5	" "	"	"
27	15	7 "	0.5	" "	"	D 8 days
28	20	7 "	0.1	" "	"	S
29	20	14 "	0.01	" "	I	"
30	20	21 "	0.01	" "	"	"
31	20	21 "	0.01	" "	"	"
32	20	21 "	0.01	" "	"	"
33	15	21 "	0.01	" "	"	"
34	20	21 "	0.01	" "	"	"

D indicates death of the animal.
S indicates survival of the animal.

TABLE III—*Concluded.*

Rabbit No.	Amount of serum	Time interval	Infection with	Result
	cc.			
35	10	1 hr.	0.1 cc. Type I	S
36	10	1 "	0.1 " " "	"
37	10	2 hrs.	0.1 " " "	"
38	10	4 "	0.5 " " III	D 7 days
39	10	4 "	0.5 " " "	S
40	10	24 "	0.1 " . " I	D 10 days
41	10	24 "	0.5 " " III	" 3 "
42	8	24 "	0.5 " " "	" 7 "
43	10	24 "	0.1 " " I	S
44	12	3 days	0.1 " " III	"
45	8	7 "	0.4 " " "	"
46	8	7 "	0.5 " " "	D 7 days
47	10	7 "	0.01 " " I	" 2 "
48	10	7 "	0.01 " " "	S
49	10	14 "	0.1 " " "	D 4 days
50	10	21 "	0.1 " " "	" 4 "
51	10	21 "	0.1 " " "	" 2 "

stances where the time interval was longer than 7 days, none of the animals recovered.

In all of the experiments controls were used consisting of rabbits which received quantities of normal whole bood or serum equal to the amount of immune blood or serum transferred. Altogether 20 control rabbits were transfused with normal whole blood and 10 received injections of normal serum. They all died of pneumococcus septicemia.

Differences in the effectiveness of passive protection depending on the time elapsing between administration of serum or blood and the injection of the infecting organism may be noted in Table III. In those instances where serum was employed, protection appeared to be most effective, if only a few hours elapsed before the injection of organisms. On the other hand, when whole blood was transfused, protection was less striking when the interval was short than when the animals were permitted to rest 24 hours or longer before infection. The duration of the protection conferred by whole blood is evidenced by animals which survived pneumococcus infection 3 weeks after transfusion.

No definite conclusions can be drawn at the present time from the results concerning the time intervals employed in passive protection tests. The significant fact is that the circulating blood of rabbits immunized with R pneumococci possesses active prinicples which, when transferred to normal rabbits, confer upon the recipients protection against infection with virulent pneumococci.

TABLE IV.

Comparison of Passive Protection of Mice and Rabbits by Serum of Rabbits Immunized with R_2 Pneumococci.

Anti-R rabbit serum		Amount of culture Type I	Results	
			Rabbits	Mice
cc.		*cc.*		
10		0.1	D 24 hrs.	
10		0.01	S	
10	*Interval of 2 hrs.*	0.01	S	
10		0.01	S	
0.5		0.00001		D 24 hrs.
0.5		0.00001		D 24 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 36 hrs.
0.5		0.000001		D 40 hrs.
		Controls		
None		0.000001	D 60 hrs.	D 24 hrs.
None		0.000001		D 24 hrs.
None		0.000001		D 30 hrs.

D indicates death and figures the number of hours before death of animal.

* 2 hours following intraperitoneal injection of serum all animals were infected intraperitoneally with virulent culture in amounts indicated.

In contrast to the effective protection of rabbits just described, attempts to protect mice by the use of the same sera have been entirely negative. From Table IV it may be seen that serum which protected rabbits against 0.01 cc. did not protect mice against even 0.000001 cc. of culture. This was true in spite of the fact that the mice received a much larger amount of serum per unit of body weight than did the ràbbits. Repeated attempts to protect mice with resistant rabbit's serum have failed regardless of whether the infecting organisms were

introduced simultaneously with serum or after intervals of 2, 6, 12, or 24 hours. The whole citrated blood of rabbits has been similarly tested and found to be without effect in mice.

It may be concluded, then, that the serum or whole blood of rabbits immunized by repeated injections of R pneumococci, although able to afford protection to normal animals of the same species, is incapable, under comparable conditions, of conferring protection upon animals of a foreign species—*i.e.* mice. These results are in striking contrast to those obtained with antipneumococcus sera which possess a high content of type-specific antibodies.

DISCUSSION.

The experiments recorded in the present communication demonstrate that a considerable degree of active immunity against Type I and Type II pneumococci may be stimulated in rabbits by repeated injections of R pneumococci. This form of resistance, elicited by R organisms which are devoid of type specificity, is effective in the absence of demonstrable *type-specific* agglutinins, precipitins, and antibodies passively protective for mice. In a preceding paper (1) it was reported that rabbits similarly treated are resistant to virulent Type III organisms; the present results with Types I and II establish the fact that immunity induced in rabbits by R strains is sufficiently broad to be effective against infection with each of the three specific types of pneumococcus. The Type III infections as previously pointed out, were characterized by a bacteremia which ran a polonged course during which the number of circulating bacteria varied from time to time but eventually disappeared. The Types I and II infections encountered in the present experiments behave similarly. These facts are suggestive that in this form of immunity the mode of recovery from infection involves the same mechanism, or different mechanisms acting in a similar manner, against each type of pneumococcus. Furthermore, these results strongly imply that resistance under these conditions is dependent either upon other factors than those concerned in type-specific immunity, or upon the same factors operative in a different manner.

Although the majority of previous investigators have found that active immunity against pneumococcus infection is type-specific, the ex-

perimental conditions, either as to the species of animal or the method of immunization employed, have differed from those reported in this paper. Cecil and Blake (6) observed that in monkeys vaccination with living cultures of Type I conferred a certain amount of cross-immunity, the degree of effectiveness being subject to variation. The immunizing dose in their experiments consisted of *one* subcutaneous injection of either 0.001 cc. of virulent or 1 to 2 cc. of avirulent organisms. Wright (7) found that *one* preliminary intravenous injection of S pneumococci produced active immunity in rabbits effective only against homologous organisms. Barach (8) employing mice gave *one* intraperitoneal injection of S organisms and obtained strict type-specific immunity.

In the experiments here reported the active resistance which was stimulated by immunization with R pneumococci and which was found to be effective against all the fixed types, was obtained by a more prolonged series of injections. The process of immunization comprised 18 intravenous injections carried out over a period of 6 weeks according to the method described by Cole and Moore (2). Although the degree of immunization necessary to incite non-type-specific immunity has not been determined, it has been found that one injection of from 5 cc. to 25 cc. of R culture is insufficient.

The purpose of these experiments has been an attempt to understand the factors underlying the resistance. Since this form of cross-immunity can be induced by pneumococci devoid of type-specific properties, it seems highly probable that a mechanism of a different order from that involved in type-specific resistance is implicated. Work is in progress at the present to define more clearly the points of similarity and difference between these two forms of acquired resistance.

Having determined the presence of active immunity in rabbits previously treated with R cells, consideration has been given to passive immunity. Since one of the chief characteristics of type-specific immunity is the passive protection afforded animals of any species by an immune serum of the homologous type a study of the possibility of passively transferring this non-specific resistance has been carried out. It was found that whole blood or serum of R immunized rabbits protected normal rabbits against infection with virulent Type I and

Type III pneumococci.* As a rule, blood in 15 to 20 cc. amounts was found to afford a more solid resistance against a given infecting dose than the equivalent amount of serum.

Under the experimental conditions described the most striking results have been obtained when an interval of time elapsed between the transfusion and the injection of organisms. The exact significance of these relations has not as yet been sufficiently studied to justify final conclusions. However, it can be stated at the present time, that the immunity elicited by repeated injections of R pneumococci in rabbits can be passively transferred by the circulating blood to normal rabbits. This is evidence that there is present in the circulation of resistant rabbits either protective substances in an active state or something which stimulates the mechanism of resistance in the transfused animal.

Attempts to confer passive protection on mice under similar conditions have been uniformly negative. This failure is in sharp contrast to the positive results always obtained in mice with type-specific sera, and is further evidence of a difference in the mechanism involved in each instance.

SUMMARY.

1. Rabbits, vaccinated by repeated intravenous injections of suspensions of heat-killed R pneumococci, acquire a marked degree of active immunity to infection with the virulent S forms of Pneumococcus Types I and II. Previously (1) it was shown that the immunization of rabbits with R cells induces active resistance to Type III infection. This immunity is effective when the infecting organisms are injected either intravenously, intraperitoneally, or intradermally.

2. Whole citrated blood or serum of rabbits immunized with R pneumococci, under the experimental conditions described, is capable of passively protecting normal rabbits against Type I and Type III infection. Whole blood appears to be more effective than an equivalent amount of serum.

3. Passive protection of mice by the use of whole blood or serum of

* Resistance to Type II was not tested by reason of the fact that an R strain originally derived from Type II S culture was used for immunization. It seemed desirable to minimize the possible participation of type-specific substances.

the immune rabbits has been entirely ineffectual. This is in striking contrast to the results obtained with type-specific immune serum.

4. This form of acquired resistance to pneumococcus infection, elicited by R organisms which are devoid of type specificity, and exemplified in animals whose sera possess no demonstrable type-specific antibodies, has many characteristics strongly suggesting that the underlying mechanism differs from that concerned in type-specific immunity.

CONCLUSION.

A broad immunity against infection with virulent S pneumococci (Types I, II, and III) can be induced in rabbits by vaccination with the degraded R strains of pneumococcus. This form of active resistance is effective in the absence of demonstrable type-specific antibodies, and may be passively transferred to normal rabbits by the blood of the immunized animal.

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SUPPRESSION OF THE FIRST ATTACK WITH SUBSEQUENT RELAPSE: AN IMMUNE PHENOMENON IN EXPERIMENTAL RELAPSING FEVER.

By HENRY EDMUND MELENEY, M.D.

(From the Department of Medicine, Peking Union Medical College, Peking, China, and the Department of Preventive Medicine and Public Health, Vanderbilt University, Nashville, Tennessee.)

A series of experiments has already been reported (1) in which it was found that Chinese squirrels and chipmunks, when inoculated intraperitoneally with *Spironema recurrentis*, would show many spirochetes in the blood for a period of 1 to 5 days, and would recover without a relapse; but if the spleen were removed one or two relapses would occur. In a subsequent paper (2), the development of six distinct strains of spirochetes as the result of relapses was reported, and it was shown that strains which appeared in alternate attacks were closely related to one another, while strains appearing in consecutive attacks were immunologically quite different from each other.

During the course of the latter experiments a phenomenon was encountered, of which I have seen no report in the literature on relapsing fever. This phenomenon was the suppression of the first attack in animals which were reinoculated with a strain of spirochetes which had recently been present in the blood, and the subsequent occurrence of a relapse after the interval which usually occurred between attacks. That this delayed attack was a relapse and not a true first attack with a long incubation period, was proven by the fact that the spirochetes causing it were shown to belong to a different strain than the strain inoculated. The phenomenon was met with five times during the course of the experiments, as shown by the following protocols.

PROTOCOLS.

Observation 1.—A squirrel whose spleen had been removed 7 days previously, was inoculated with Strain I spirochetes. The tail blood was positive for the 4 succeed-

ing days, but no relapse occurred. After this attack the blood serum showed the presence of agglutinins only for Strain I. 32 days later the squirrel was reinoculated with Strain I. For 6 days thereafter no spirochetes appeared in the tail blood; but on the 7th day they appeared, and continued to be present in greatly increasing numbers for 4 days. After this attack the blood serum con-

TABLE I.

Course of Infection in Squirrel of Observation 1.

Date	Strain inoculated	Attacks			Negative intervals, duration	Titer of serum during interval for strain					Strain causing attack
		No.	Day	Tail blood		I	II	III	IV	V	
					days						
IV/1/27	I	1	1 2 3 4	+ ++ +++ ++++	32	1280	0	0	0	—	I
V/7/27	I			Neg.	6						I
		2	1 2 3 4	+ ++ ++++ ++++	7	100+	100+	0	0	0	II
		3	1 2 3 4 5	+ + +++ +++ +++	6	100+	100+	100+	0	0	III
		4	1 2 3 4	+ ++ ++ +	20						V*

* Origin of Strain V. Proved by agglutination tests not to belong to Strain I, II, III or IV.

tained agglutinins for both Strain I and Strain II, indicating that the spirochetes causing the delayed attack were not the strain inoculated but belonged to Strain II, the strain which usually followed Strain I in the first relapse. After another interval of 7 days another relapse occurred, which caused the development of additional agglutinins for Strain III. Another interval of 6 days then occurred, followed by another relapse. The spirochetes of this relapse were tested against

monovalent immune sera and were found to belong to Strain V (see Meleney (2) for details of strains). This squirrel, therefore, theoretically had four attacks following its reinoculation, but the first attack was suppressed because the animal was already immune to the strain of spirochetes inoculated. The record of this squirrel is summarized in Table I.

Observation 2.—A chipmunk was inoculated with Strain I spirochetes without previous splenectomy. Spirochetes were present in the tail blood on the 3 succeeding days. No relapse occurred. The blood serum after this attack contained agglutinins for Strain I but not for Strain II. Other strains were not tested. 12 days after the close of the attack the spleen was removed and 5 days after that the animal was reinoculated with Strain I spirochetes. The tail blood was negative for the next 5 days, after which an attack occurred which lasted 4 days. After this attack the animal's blood serum contained agglutinins for both Strain I and Strain II, indicating that the attack had been due not to the Strain I spirochetes with which it had been reinoculated, but to Strain II.

Observation 3.—A second chipmunk was inoculated with Strain I spirochetes without previous splenectomy. The tail blood was positive for spirochetes on the 3 succeeding days. No relapse occurred. The spleen was removed 16 days later, and 14 days after splenectomy the animal was reinoculated with Strain I spirochetes. The tail blood remained negative for 5 days, after which an attack ensued with spirochetes in the blood for 5 days. After this the animal's serum was found to contain agglutinins for Strain II, indicating that the attack had been due to that strain rather than to Strain I, with which the animal had been reinoculated. After another interval of 8 days a second relapse occurred lasting 2 days.

Observation 4.—A squirrel was inoculated on the day following splenectomy with Strain II spirochetes. After an incubation period of 3 days spirochetes appeared in the blood for 2 days. The attack was followed by a negative interval of 7 days and a relapse lasting 3 days. There was no second relapse. The animal's serum after the relapse contained agglutinins for Strain II, Strain I and Strain III.[1] After waiting 20 days from the close of the relapse, this squirrel was reinoculated with Strain III spirochetes. A negative interval of 6 days ensued, and then spirochetes appeared in the blood for 3 days. After this attack the animal's serum contained additional agglutinins for Strain V, indicating that the attack had been due to that strain rather than to Strain III, with which it had been reinoculated. After another interval of 4 days a relapse occurred in which the spirochetes were proven by agglutination tests to belong to Strain VI.

Observation 5.—This squirrel was inoculated three different times. The first inoculation was made with Strain I on the day following splenectomy. A single attack followed which lasted 5 days. After this attack the animal's serum contained agglutinins only for Strain I. After waiting 23 days it was reinoculated with Strain I and the tail blood contained spirochetes for the 3 following days.

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[1] It was frequently found that, after a first relapse in an animal inoculated with Strain II, agglutinins for both Strain I and Strain III were present.

Thus the first reinoculation was not followed by suppression of the first attack as in the other four observations. After an interval of 7 days a relapse occurred lasting 3 days, and after another interval of 9 days a second relapse occurred lasting 3 days. The spirochetes of this second relapse were proven by agglutination tests with monovalent sera to belong to Strain III, but after this relapse the animal's serum contained agglutinins not only for Strains I, II and III, but also for Strain IV. 16 days later it was reinoculated with Strain IV spirochetes. The tail blood was negative for 8 days, after which spirochetes appeared for 3 days, and were found by agglutination tests to belong to Strain V.

COMMENT.

It is apparent from the above protocols that the reason that the "first attack" failed to appear following the reinoculation in these animals, was that they were immune to the strain of spirochetes with which they were reinoculated. The spirochetes which were injected into the immune animal found themselves in an unfavorable environment. Most of them were destroyed, but a few survived because they were able to adjust themselves to the environment. In other words, the course of the disease in these animals from the time of the reinoculation was the same as that in animals which have just reached the crisis of their first attack, when the blood still contains spirochetes and the immune bodies against those spirochetes have already begun to appear. In such animals the spirochetes *in the blood stream* are rapidly destroyed, causing the first attack to stop abruptly. In the reinoculated animals the first attack is entirely suppressed by the destruction of the spirochetes *in the peritoneal cavity.* In both types of animals a few spirochetes survive and undergo a biological change into a new strain. About 6 days is required for the spirochetes of the new strain to become numerous enough to be found in the peripheral blood. This is the negative interval, and it is followed by the relapse due to the new strain of spirochetes.

This phenomenon is an additional demonstration of the immunity to a single strain of *S. recurrentis* which is conferred by an attack due to that strain. It also emphasizes the limitation of such immunity to the strain or strains which have been present in preceding attacks or to closely related strains.

In some papers on experimental relapsing fever (3), long incubation periods are described in mice which have been reinoculated with the

same strain as that used originally. It may be that these cases are examples of the phenomenon here described. (See Kudicke and Feldt, Tables 2, 5 and 6.) The present observations emphasize the importance of determining the identity of the strain of relapsing fever spirochetes which appears in any attack other than one immediately following inoculation, if reliable conclusions are to be drawn as to the immunological reactions which take place between this parasite and its host. The strain of each relapse is a strain which was not present in the animal's blood in a previous attack. In order to distinguish between a prolonged incubation period and a suppressed first attack followed by a relapse, it is necessary either to test the spirochetes causing the attack, against monovalent immune sera, or to determine, after the close of the attack, what additional agglutinins have been added to the animal's serum as a result of the attack.

SUMMARY.

In five splenectomized squirrels and chipmunks which were reinoculated with a strain of *Spironema recurrentis* which had previously been present in their blood, the first attack was entirely suppressed because the animals were immune to the strain of spirochetes inoculated; but after the interval which usually occurred between attacks, a relapse ensued, in which the strain of spirochetes present in the blood was different from the strain inoculated.

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OBSERVATIONS ON FLAGELLAR AND SOMATIC AGGLUTINATION.

By JOHN B. NELSON, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

In a recent review Hadley (1) presented a critical discussion of the flagellar antigen concept as originally proposed by Smith and Reagh (2) to account for the difference in agglutination between motile and non-motile strains of the hog cholera bacillus. He questioned the validity of the hypothesis on the ground that the particular order of serologic reaction was not limited to bacterial species characterized by the possession of flagella. He suggested that such serologic differences might eventually come to be studied in terms of the presence or absence of certain specific soluble substances wherever their point of origin in the bacterial cell. Some work done by the writer on the agglutinability of deflagellated motile bacteria seemed to have a bearing on the latter suggestion. The work was extended somewhat and is discussed in the present paper.

The initial work of Smith and Reagh (2) was concerned with the agglutinative affinities of two strains of the hog cholera bacillus, one motile, the other a non-motile variant. A "motile" antiserum agglutinated the motile strain in high dilution with a floccular type of clump. Microscopically the clumped bacilli were separated by narrow spaces. It agglutinated the non-motile strain in a lower dilution with granular clumps in which the bacteria were not separated by any appreciable space. A "non-motile" antiserum agglutinated both strains in relatively low dilution with granular clumping. The non-motile strain removed the granulating but not the flocculating agglutinin from "motile" antiserum. The motile strain reduced the granulating agglutinin of the "non-motile" serum. The writers concluded that the agglutinins for the flagella and for the body of the bacilli, at least so far as the large group of pathogenic colon derivatives were concerned, were distinct, not mutually interacting substances.

These observations were subsequently extended by other workers among whom the following may be mentioned. Beyer and Reagh (3), working under Smith,

showed that flagellar antigen and somatic agglutinin were heat-labile at 70°C. while somatic antigen and flagellar agglutinin were heat-stable. Orcutt (4) working with the hog cholera bacillus and Balteanu (5) with the vibrio comma demonstrated the agglutinative and antigenic properties of pure flagellar suspensions. Yokota (6) carried out similar studies on flagellar suspensions from *B. typhosus* and also on the bacteria deflagellated by shaking. Goyle (7) compared the agglutinative relationships of heated and untreated suspensions of *B. typhosus* and *B. enteritidis* and their variants. Arkwright (8) described the microscopic findings with floccular and granular agglutination.

The multiple antigen hypothesis as related to bacteria has also been criticized by Tulloch (9) in an extended series of papers. He pointed out certain irregularities which cannot be cited here.

Direct Agglutination of Whole, Shaken and Heated Antigens by "Whole" Antiserum.

Two species of Salmonella designated *B. paratyphi* Types I and II were employed in the present work.

The strains used were originally cultivated from spleen tissue. They were isolated during the course of a natural epidemic in a guinea pig population (10). The final cultures represented the growth from single colonies replated three times on agar. They were normal smooth strains as to their growth in broth and their colony formation on agar, were actively motile and gave a characteristic agglutination with diagnostic antiserums then in use.

Fresh antiserums against the whole bacteria were prepared in rabbits.

The growth from 18 hour agar cultures was removed with physiological salt solution, centrifuged, the packed bacteria washed once, resuspended in 0.2 per cent formalinized saline and heated to 56°C. for 1 hour. Five intraperitoneal injections at 3 day intervals were given. The rabbits were previously tested for normal or immune agglutinins active for the particular antigens. The two type antiserums, termed "whole," were tested against whole, heated and shaken suspensions of both types of *B. paratyphi*. The suspensions were prepared as follows: The growth from a Blake bottle was removed with 10 cc. of saline, washed once, resuspended in saline and divided into three portions. One was untreated. One was heated to 100°C. for 30 minutes in a water bath, washed once after centrifuging and resuspended in saline. One was shaken for 1 hour in a mechanical shaker, washed twice after centrifuging and resuspended in saline. The three suspensions were finally standardized to equal opacity, 2.4 on the Gates scale. In testing, 0.5 cc. amounts of diluted serum and suspension were mixed. The final dilutions were doubled at each interval from 1:100 through 1:51,200. The

tubes were incubated at 37°C. for 3 hours and read after overnight refrigeration. The limits of agglutination with each serum against the six antigens are given in Table I. The limit of agglutination is defined as the highest dilution showing any macroscopic evidence of clumping after approximately 24 hours.

The agglutination of the several antigens with reference to the character of the sediment and the limiting dilution was much the same with both antiserums. The whole antigen was agglutinated in high dilution by its homologous serum. The heated and shaken antigens were agglutinated equally through a much lower limit. With the

TABLE I.

Direct Agglutination of Whole, Shaken and Heated Antigens with Type I and Type II Antiserums.

Serum	Antigen	Limiting dilution	Type of agglutination
Type I "whole"	Type I whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" II whole	1:800	Atypical granular
	" " shaken	1:800	Granular
	" " heated	1:800	"
Type II "whole"	" II whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" I whole	1:200	Atypical granular
	" " shaken	1:200	Granular
	" " heated	1:200	"

antiserums of opposite type the three antigens were agglutinated equally in still lower dilution. The reaction with the homologous whole antigen was predominantly floccular throughout with an admixture of granular clumps in the lower dilutions. The reaction was rapid with the formation of coarse clumps which on settling formed a light feathery sediment. In the lower dilutions the supernatants were clear or nearly so. Microscopically the sediment was composed mainly of large, poorly defined clumps with an open work appearance. With the lower dilutions there were also smaller compact clumps which were more sharply defined. The type of agglutination with the ho-

mologous heated and shaken antigens was granular throughout. The reaction was retarded. The sediment varied from an irregular, wrinkled, compact disc in the lower dilutions to a granular mold fitting the rounded portion of the tube in the higher dilutions. The clumps were not easily broken up on shaking. The supernatants tended to be clear up to the last two dilutions with a graded sediment. With the antigens of the opposite type the agglutination was granular. The macroscopic picture with the heated and shaken antigens was identical with that described above. The character of the sediment with the whole antigens was somewhat different. The Type II antigen formed a compact, even, button-like sediment with a practically clear supernatant. The Type I antigen formed a sediment closely approaching the typical granular form except that the clumps were larger and not as closely packed. Microscopically the clumps were of the granular type in both cases.

Absorption of "Whole" Antiserum with Whole, Shaken and Heated Antigens.

The two "whole" antiserums were absorbed with the three homologous antigens.

In absorbing 0.25 cc. of packed bacteria, 0.1 cc. of serum and 2.4 cc. of 0.2 per cent formalinized saline were employed, an absorbing dose and a serum dilution of approximately 1:10 and 1:25 respectively. The absorbing antigens were prepared as previously described and sedimented for 1 hour in graduated centrifuge tubes to a constant volume. The final antigen-serum mixtures were incubated at 37°C. for 3 hours with frequent shakings. After overnight refrigeration they were centrifuged and the clear supernatants tested. The dilution series previously described was employed. Unabsorbed serum similarly diluted with formalinized saline was subjected to the same incubation and tested with the same antigen suspension. The results were identical with those given in Table I. The limiting agglutination of the three antigens by the absorbed serums is given in Table II.

After absorption with whole antigen both serums continued to give a floccular reaction with the homologous antigen in very low dilution. There was no reaction with the shaken and heated antigens in the lowest dilution employed. Absorbed with shaken and heated antigens both serums agglutinated the homologous whole antigen through dilution 1:12,800, one dilution removed from the limit of agglutina-

tion with the unabsorbed serum. The type of clumping was purely floccular in all cases. The supernatants showed a diffuse turbidity which was graded. There was no agglutination of the shaken and heated antigens in the lowest dilution. The removal of a considerable amount of flocculating agglutinin by the treated antigens was indi-

TABLE II.

Agglutination Limits with Type I and Type II Antiserums after Absorption with Whole, Shaken and Heated Antigens.

Serum	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I whole	Type I whole	1:400 <1:100 <1:100	Whole Shaken Heated
	Type I shaken	1:12,800 <1:100 <1:100	Whole Shaken Heated
	Type I heated	1:12,800 <1:100 <1:100	Whole Shaken Heated
Type II whole	Type II whole	1:200 <1:100 <1:100	Whole Shaken Heated
	Type II shaken	1:12,800 <1:100 <1:100	Whole Shaken Heated
	Type II heated	1:12,800 <1:100 <1:100	Whole Shaken Heated

cated. Further evidence bearing on the absorption of flocculating agglutinin by the treated antigens will be presented in another paper.

Agglutination with Shaken and Heated Antiserums before and after Absorption.

The immunizing properties of the shaken and heated suspensions of the motile bacteria were determined.

The suspensions were prepared as described. The treated and washed bacteria were sedimented in the centrifuge, resuspended in 1 per cent formalinized saline and kept for 3 days in the refrigerator. They were resedimented, washed twice and resuspended in 0.2 per cent formalinized saline. It was necessary to kill the shaken bacteria and the above method was chosen in preference to heat at 56–58°C. on successive days. To keep the suspensions uniform both were subjected to the same conditions although the heated suspension (100°C. for 30 minutes) was obviously inactivated. Subcultures made on consecutive days were sterile. A rabbit was immunized intraperitoneally with each suspension. Five injections at 3 day intervals were given with the Type I antigens, four injections with the Type II. 7 days after the last injection the animals were bled and killed. At autopsy no focal lesions were detected in any of the usual sites: spleen, liver or lymphoid tissue of the intestinal tract. There were traces of exudate on one or another of the visceral organs. Walled-off accumulations of purulent material along the cecum were commonly observed. Cultures from spleen, liver, purulent material and cecal feces failed to show *B. paratyphi*. The agglutinin content of the several antiserums was determined by direct agglutination and by absorption employing whole, shaken and heated antigens of the same type. The limits of agglutination of the shaken and heated antiserums before and after absorption are given in Table III.

The shaken and heated antiserums agglutinated the three homologous antigens equally. The limiting dilutions were higher with the Type I serums as might be expected in view of the longer immunization. In both cases the clumping of the shaken and heated antigens was typically granular. The sediment with the whole antigens was granular microscopically. Macroscopically the sediment with each type was identical in appearance with that described previously for whole antigen agglutinated by serum of opposite type.

The absorption tests gave further evidence for the absence of flocculating agglutinin. Absorption with any one antigen removed most of the agglutinin for all three suspensions. Irregularities in the titer were noted but had no bearing on the presence or absence of flocculating agglutinin. In general the reduction of agglutinin was a little less complete than that previously noted with the whole antiserums. Although the shaken and heated antigens were able to remove some flocculating agglutinin from whole antiserum, there was no indication that they were able to produce the same upon injection in rabbits.

TABLE III.

Agglutination Limits with Shaken and Heated Serums of Both Types before and after Absorption.

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I shaken	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
Type I heated	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:100	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:100	" " heated
Type II shaken	1:3,200	Type II whole	Type II whole	<1:100	Type II whole
				<1:100	" " shaken
				<1:100	" " heated
	1:3,200	Type II shaken	Type II shaken	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
Type II heated	1:3,200	Type II whole	Type II whole	1:200	Type II whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II shaken	Type II shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated

Agglutination of Untreated, Shaken and Heated Suspensions of a Non-Motile Bacterium.

The agglutination tests were repeated with a culture of *Staphylococcus aureus* and its homologous antiserum to afford a basis for comparing the agglutinative affinities of a non-motile organism. The staphylococcus was recently isolated from a skin abscess and was a normal, smooth strain. A rabbit antiserum against the whole antigen was prepared. The same procedures outlined for the motile bacteria were followed in immunization, preparation of antigens and performance of the agglutination tests. The limits of agglutination before and after absorption with whole and heated antigens are given in Table IV.

TABLE IV.

Limits of Agglutination with Staphylococcus Antiserum before and after Absorption

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Staphylococcus whole	1:1,600 G*	Whole	Whole	1:50 G	Whole
			"	1:50 G	Heated
	1:1,600 G	Heated	Heated	1:50 G	Whole
			"	1:50 G	Heated

* Granular clumping.

The shaken antigen showed the same limiting dilution upon direct agglutination and was not employed in absorption.

The staphylococcus antiserum agglutinated whole and heated antigens to the same titer limit. The reactions were retarded, the limiting dilutions were relatively low and the clumping was typically granular both macroscopically and microscopically. The titer of the serum, 1:1,600 after five injections, was not noticeably increased by three additional injections. The agglutination of whole and heated antigens by the "non-motile" antiserum was identical in character with that of heated and shaken antigens by the "motile" antiserums. The whole and heated staphylococcus antigens possessed approximately the same absorptive capacity for agglutinin as indicated by partial absorption. . Reciprocal absorption with a "heated" staphylococcus antiserum was not made.

The Origin of Soluble Material Extracted by Heat from the Motile Bacteria.

Some additional experiments bearing on the production of soluble material from the motile bacteria may be cited. A considerable decrease was noted in the volume of packed bacteria after heating to 100°C. for 30 minutes. Goyle (7) had previously called attention to the decreased opacity of heated bacterial emulsions. The supernatants from such heated suspensions after sedimentation of the bacteria were distinctly milky in appearance and when mixed with "whole" antiserum gave a precipitate in moderate dilution. Specific soluble material precipitable with immune serum had evidently been extracted from the bacteria at the temperature employed. Happold (11) has recently described a precipitinogen present in the filtrates of steamed broth cultures of *B. aertrycke* (mutton).

Comparative tests were made on the supernatants from heated whole suspensions and heated shaken suspensions to determine the effect of the presence of flagella on the amount of extracted soluble material. Filtrates of the growth from 18 hour agar cultures of the two motile bacteria were included.

Blake bottles were inoculated with 2.5–3 cc. of 6 hour broth cultures of the bacteria and incubated at 37°C. for 18–24 hours. The growth from each bottle was removed with 5 cc. or so of saline, transferred to a graduated tube and centrifuged for 1 hour. The supernatant was withdrawn and the packed bacteria washed once with 8 cc. of saline. Both supernatants were saved and later filtered through medium Berkefeld candles. For the final suspensions 1 cc. of saline was added for each 0.1 cc. of packed bacteria and the cells resuspended. One suspension of each type was shaken for 1 hour. The packed bacteria, after centrifuging, were washed three times and finally resuspended in saline according to the same ratio. The untreated and the shaken suspensions of each type were then heated to 100°C. for 1 hour in a water bath. After centrifuging the supernatants were removed and filtered through Berkefeld filters.

The final solutions prepared according to the above methods all had a milky, opalescent appearance. There was no deposit upon standing. The culture supernatants were perfectly clear but showed a yellowish tinge. The volume of packed bacteria before heating was approximately 1 cc. with Type I, 0.7 cc. with Type II. After heating, the volumes were 0.7 cc. and 0.4 cc. respectively. The suspensions were centrifuged for 1 hour at the same speed in both cases. The culture and suspension filtrates were tested with "whole" antiserum of the same type. Only one culture filtrate was employed with the Type II *B. paratyphi*.

In testing, the antigens were diluted in series and the serum kept constant, 0.5 cc. amounts of the former and 0.1 cc. amounts of the latter being employed. The tubes were incubated at 37°C. for 1 hour, followed by overnight refrigeration. The final readings are given in Table V. The first culture filtrates and the suspension filtrates showed a ring reaction with beginning precipitation in the lowest dilutions after 1 hour. Upon standing there were decreasing amounts of a granular sediment, indicated in the table by plus signs. The highest dilutions showed a distinct clouding of the supernatant but no sediment. No bacteria were detected upon microscopic examination.

TABLE V.

Precipitin Tests with Culture Filtrates and Heated Suspension Filtrates of the Two Salmonella Types.

Antigen	Serum	Serum dilutions								
		1:5	1:10	1:20	1:10	1:80	1:160	1:320	1:610	1:1,280
Type I 1st culture filtrate 2nd " " Heated whole suspension " shaken "	Type I whole	+ S.cl. ++ ++	+ — ++ ++	± — + +	Cl.* — + +	S.cl.* — ± ±	— — ± ±	— — Cl. Cl.	— — S.cl. S.cl.	— — — —
Type II 1st culture filtrate Heated whole suspension " shaken "	Type II whole	+ ++ ++	± ++ ++	± ++ ++	S.cl. + +	— ± ±	— ± ±	— Cl. Cl.	— S.cl. S.cl.	— — —

* Cl. = cloudy supernatant; S.cl. = slightly cloudy supernatant.

The above tests indicate the presence of small amounts of soluble precipitate material in the diluted culture filtrates of both Salmonella species. The amount was greatly reduced by one washing as indicated by the reaction with the second Type I culture filtrate. Similar material was extracted from the bacteria by heat and was present in considerably greater amount in the diluting medium. The supernatant from the heated whole bacteria reacted quantitatively the same as that from the heated shaken bacteria. The amount of soluble material present in the fluid as a result of flagellar disintegration was evidently too small to affect the titer. It may be said, too, that the

agglutinability of the bacteria was not greatly influenced by the loss of the somatic constituents extracted by heat.

DISCUSSION.

The experimental work on agglutination reported in the previous section is in no sense original. The general conformation of the observations to the early flagellar hypothesis of Smith and Reagh (2) and its extension by others is the main reason for its presentation. The observations bear out the altered agglutinability of motile bacteria following shaking and heating at 100°C. Both treatments result in deflagellation of the bacteria. With the former the flagella are mechanically broken off and subsequently removed by washing. With the latter a disintegration of the flagella occurs and the soluble products are removed upon washing. Tulloch (9) has objected to the application of heat for demonstrating separate antigens on the ground that the physical nature of the bacterial cell as a whole may undergo a change resulting in altered agglutinability. The results following deflagellation by shaking are less easily disposed of in this way. No marked change in the physical state of the cell is apparent, aside from the removal of one morphological structure, the flagellum. The shaken bacteria are viable and capable of normal growth upon transfer to media. In common with the heated bacteria, however, they are incapable, in the present case, of producing flocculating agglutinin upon injection in rabbits. Admittedly there is lack of agreement concerning the true antigenic nature of deflagellated bacteria. In some instances at least the reported ability of treated bacteria to produce flocculating agglutinin appears referable to the presence of flagellar material in the suspension used for immunization.

One exception to the flagellar hypothesis was noted with the present work. The shaken and heated suspensions were able to absorb a certain amount of flocculating agglutinin from "whole" antiserum. It is suggested that the anomalous reaction may be attributed to a non-specific adsorption of antibody. Regarding the reaction in an animal host as the more exacting criterion it appears that the motile bacteria have lost in immunizing ability with the loss of flagella. This in turn implies that the substance of the flagella embraces a specific antigen.

The objections of Hadley (1) to such an assumption have been previously noted. He intimates that serological differences similar to those noted with whole and deflagellated bacteria may be explained by the presence of soluble specific substances disregarding particular morphological elements.

A small amount of soluble material precipitable with immune serum was found present in culture medium supporting the growth of the motile bacteria. The suspensions used for the production of "whole" antiserum were carefully washed and the amount of such material reduced to a practically negligible quantity. The resulting antiserum, however, contained both granulating and flocculating agglutinin. If a soluble substance were responsible for the appearance of flocculating agglutinin the bulk of it must have been produced from the bacteria after their introduction into the animal host. The "shaken" and "heated" antiserums contained only granulating agglutinin. Hence if a soluble substance is to be regarded as the antigen leading to the production of flocculating agglutinin it seems necessary to associate it with the flagella. Orcutt (4) has shown that pure flagellar suspensions (hog cholera bacillus) give a floccular agglutination with "whole" or flagellar antiserum. If the suspension is heated to 70°C. for 30 minutes the flagella are broken up. The floccular agglutinability is lost but on animal injection the heated suspension produces flocculating agglutinin. Apparently the flagella go into solution. With our experiments such heated suspensions in moderate dilution (1:80) gave a granular precipitate with "whole" antiserum. One might regard this reaction as the analytical production of soluble specific material which was antigenic in the sense that it could still produce antibody but which because of a change in the physical state of its precursor, the flagellum, showed altered agglutinability. Injected bacteria must eventually undergo disintegration probably with the production of similar soluble materials. Viewed in this light the suggestion of Hadley seems plausible with this modification, however, that the soluble substance be limited as an antigen to the flagella.

Hadley (1) has also questioned the purity of the flagellar suspensions used by Orcutt (4) and Balteanu (5). He suggests that the heat-labile factor present in such suspensions may equally well represent

soluble substances from the bacteria themselves. We have shown that the bulk of soluble precipitable material extracted *in vitro* from motile bacteria or produced *in vivo* in smaller amounts during growth originates in that portion of the bacterial cell which as an antigen gives rise to granular agglutinin. In combination with "whole" antiserum this material gives a precipitate which is likewise granular in nature. Its presence in flagellar suspensions could not be held accountable for the floccular reaction which occurs with "whole" or flagellar antiserums.

SUMMARY.

Whole, shaken and heated suspensions of two Salmonella species were compared as to agglutinability, absorptive capacity and antigenic properties. The results were in general agreement with the flagellar antigen concept of Smith and Reagh. The removal of flagella by shaking or heating (100°C.) resulted in altered agglutinability manifested by failure to give a floccular reaction with "whole" antiserum. The deflagellated bacteria were able to absorb some flocculating agglutinin from that serum. They were unable, however, to produce flocculating agglutinin upon injection in rabbits.

Untreated, shaken and heated suspensions of a non-motile bacterium (*Staphylococcus*) showed no differences with respect to agglutinability or absorptive capacity.

Soluble precipitable material was found present in small amount in culture filtrates of the motile bacteria and in greater concentration in filtrates of heated suspensions. The bulk of the soluble material was of somatic origin and was not appreciably increased by the presence of flagella. It was possible, however, to demonstrate soluble material in heated flagellar suspensions. The relation of such soluble substances to floccular agglutination and the production of flocculating agglutinin as suggested by Hadley is discussed.

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THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA.

By JOHN B. NELSON, Ph.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

The flagellar antigen hypothesis of Smith and Reagh (1) assumes that motile bacteria as antigens give rise to two distinct types of agglutinin, termed flagellar and somatic agglutinin respectively. The former is produced by the flagella and reacts with them, whether attached to the cell or free, to give a floccular agglutination. The latter is produced by the bacterial cell proper and reacts with whole or deflagellated bacteria to give a granular agglutination. The somatic antibody does not combine with flagella. Some experiments on the agglutinability of whole and deflagellated suspensions of motile bacteria were reported in a preceding paper (2). The performance of absorption tests, during the course of the work, suggested that it might be possible to demonstrate differences between the two agglutinins with respect to their removal from sensitized bacteria.

A number of methods have been employed with more or less success in attempts to remove antibody from combination with sensitized bacteria. The early work has been reviewed in some detail by Huntoon (3). In another paper Huntoon and Etris (4) have reported an extensive series of experiments designed to secure purified solutions of antibody, largely by removal from sensitized antigens. They worked with non-motile bacteria chiefly the pneumococcus but also the meningococcus and *B. dysenteriæ* Flexner. They showed that agglutinin could be removed by suspending the sensitized antigen in 10 per cent saccharose solutions or distilled water and heating to 55–60°C. for 1 hour. They were able to remove as high as 50 per cent of the combined antibody. They found that the presence of sodium chloride interfered with the removal of agglutinin but not with the removal of bactericidal and protective antibodies. A temperature of 65°C. reduced the dissociation of antibody and 70°C. almost completely prevented it. Other experiments reported by Huntoon and Etris on the removal of protective antibody from sensitized pneumococcus have no immediate bearing on the present work.

The close association of antibodies with serum globulins suggested the application of some method of globulin extraction for the removal of agglutinin. In some preliminary work it was found that heating sensitized motile bacteria to 60°C. in the presence of 5 per cent sodium chloride solution resulted in the removal of a considerable amount of one agglutinin but little or none of the other. More detailed observations on the application of the method are reported in the following experiments.

Two species of *B. paratyphi* (Types I and II) (5) of guinea pig origin were employed in the absorption and extraction tests. The serums were from rabbits immunized with inactivated suspensions of the whole bacteria.

In the absorption tests a serum dilution of approximately 1:25 and an absorbing dose of 1:10 were employed. The bacterial suspensions were freshly prepared, washed once and centrifuged to constant volume—0.25 cc. 0.1 cc. of "whole" antiserum and 2.4 cc. of physiological salt solution were added and the packed bacteria resuspended. The absorption mixtures were incubated for approximately 5 hours at 37°C. followed by overnight refrigeration. Unabsorbed serum in the same dilution was included as a control. The sensitized bacteria were sedimented in the centrifuge and the clear supernatants saved. The packed bacteria were washed four times by resuspending in 5 cc. of saline and centrifuging. After the fourth washing the packed bacteria were resuspended in 5 cc. of 5 per cent sodium chloride solution and heated to 60°C. for 1 hour in a water bath. The mixture was shaken at frequent intervals. After a final centrifuging the supernatants were withdrawn. In several instances second and third extractions were carried out in the same manner.

The agglutinin titer of the several supernatants was determined by macroscopic agglutination tests. 0.5 cc. amounts of diluted test fluid and of bacterial suspension were employed. Whole and heated suspensions were used as antigens. The former was a fresh, untreated culture, washed, and diluted to standard opacity with saline. It represented both flagellar and somatic antigens. The latter was a fresh suspension heated to 100°C. for 30 minutes, washed and similarly diluted. It represented pure somatic antigen. The tubes were incubated for 3 hours at 37°C. and read after overnight refrigeration.

The agglutination readings with unabsorbed serum, absorbed serum, four washing fluids and three extraction fluids are given in Table I. The work was done with Type II *B. paratyphi* and its homologous "whole" antiserum.

The unabsorbed serum agglutinated the whole antigen through a

TABLE I.

Agglutination of B. paratyphi Type II by "Whole" and Absorbed Serum, Washings and Extraction Fluids.

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	61	128	256	512	1,021	2,018
Unabsorbed serum	W*	CM	C	C	C	++++	++++	+++	+++	++	+	—
	II	CG	C	++++	+++	++	+	±	—	—	—	—
Absorbed serum	W	+++M	+	±	—	—	—	—				
	II	±G	—	—	—	—	—	—				
1st washing	W	+F	±	—	—	—	—	—				
	II	—	—	—	—	—	—	—				
2nd washing	W	±F	±	—	—	—	—	—				
	II	—	—	—	—	—	—	—				
3rd washing	W	±F	—	—	—	—	—	—				
	II	—	—	—	—	—	—	—				
4th washing	W	±F	—	—	—	—	—	—				
	II	—	—	—	—	—	—	—				
1st extraction	W	+++F	+++	+++	+++	+++	++	++	++	+	—	—
	II	—	—	—	—	—	—	—				
2nd extraction	W	+++F	+++	++	++	+	±	—				
	II	—	—	—	—	—	—	—				
3rd extraction	W	+++F	+	±	—	—	—	—				
	II	—	—	—	—	—	—	—				

* M = mixed agglutination; F = floccular agglutination; G = granular agglutination; W = whole antigen; II = heated antigen.

dilution of 1:1,024, the heated antigen through 1:128. The actual titer limits of the serum were 1:25,600 and 1:3,200 respectively, since an initial dilution of 1:25 was used. With the whole antigen the clumping was predominantly floccular with an admixture of granular clumps in the lower dilutions. With the heated antigen the clumping was purely granular. After absorption with the whole antigen there was a residual agglutination in low dilution. The bulk of the two agglutinins had combined with the bacteria. The first washing fluid slightly agglutinated the whole antigen in low dilution. There was no reaction with the heated antigen. The other three washing fluids in spite of progressive dilution continued to give a slight floccular reaction with whole but not with heated antigen. The first extraction supernatant gave a reduced but perfectly definite agglutination purely floccular in type through a dilution of 1:512 with the whole antigen. For comparison with the agglutinin titer of undiluted serum the initial dilution of the absorbed serum must be considered, giving a final dilution of 1:12,800. There was no reaction with the heated antigen in any dilution. A second and third extraction gave decreasing floccular agglutination with the whole antigen but no reaction with the heated.

The persistent agglutination with the washing fluids suggested that a small amount of flocculating agglutinin might be removed at room temperature. Increasing the temperature and salt content of the suspending fluid gave a marked increase in the amount removed. The method of extraction failed to remove a detectable amount of granulating agglutinin.

The above experiment was repeated with different strains of the same bacterium and with another species of Salmonella, *B. paratyphi* Type I. The results were always much the same. The limiting dilutions of the absorbed serum, washing fluids and extraction fluids varied somewhat. In several instances the titer of the first extraction fluid was one dilution lower. Sometimes, too, there was a slight granular sediment in the lowest dilution of the heated antigen series. The substitution of suspensions deflagellated by shaking instead of by heat gave identical results. Adequate controls were included for the effect of 5 per cent sodium chloride solution on whole antigen in the absence of serum and on heated antigen in the presence of

serum. There was no indication of spontaneous agglutination or of inhibition of granular clumping.

The extraction experiment was repeated with a sensitized non-motile bacterium. A strain of *Staphylococcus aureus* and its homologous antiserum were employed. The results are given in Table II. The unabsorbed serum agglutinated both antigens to the same titer limit, 1:1,600, with a granular type of clump. There was a slight residual agglutination after absorption with the untreated suspension.

TABLE II.

Agglutination of Staphylococcus by Unabsorbed and Absorbed Serum, Washing Fluid and Extraction Fluid.

Test fluid	Antigen	Test fluid dilutions							
		2	4	8	16	32	64	128	256
Unabsorbed serum	W*	CG	++++	++++	+++	++	+	—	—
	H	CG	++++	+++	++	+	±	—	—
Absorbed serum	W	±G	—	—	—	—	—	—	—
	H	±G	—	—	—	—	—	—	—
1st washing	W	—	—	—	—	—	—	—	—
	H	—	—	—	—	—	—	—	—
1st extraction	W	—	—	—	—	—	—	—	—
	H	—	—	—	—	—	—	—	—

* W = unheated antigen; H = heated antigen; G = granular agglutination.

The washing and extraction fluids failed to agglutinate either antigen in any dilution.

The extraction method was also applied to sensitized suspensions of the two motile bacteria previously deflagellated by shaking and heating respectively. The removal of some flocculating agglutinin from "whole" antiserum by such suspensions was noted in a preceding paper (2). The absorptions together with a control of unabsorbed serum were carried out in 0.2 per cent formalinized saline. Otherwise the method of procedure was identical with that previously outlined. The results for *B. paratyphi* Type II and its homologous "whole" antiserum are given in Table III.

TABLE III.

Agglutination of Shaken and Heated Suspensions of B. paratyphi Type II by "Whole" and Absorbed Serum, Washing and Extraction Fluids.

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	CM	C	C	++++	++++	+++	+++	+++	++	+	—
	SH	CG	C	++++	+++	++	+	±	—	—	—	—
	H	CG	++++	+++	++	+	±	±				
Absorbed with shaken suspension	W	++++F	++++	+++	++	+++	+++	+++	++	+		
	SH	—	—	—	—	—	—	—				
	H	—	—	—	—	—	—	—				
Absorbed with heated suspension	W	+++F	+++	+++	+++	+++	+++	+++	++	+	—	—
	SH	—	—	—	—	—	—	—				
	H	—	—	—	—	—	—	—				
3rd washing of shaken suspension	W	±F						—				
	H	—						—				
1st extraction of shaken suspension	W	+++F	+++	+++	++	++	+	—				
	H	—	—	—	—	—	—	—				
3rd washing of heated suspension	W	+F	±					—				
	H	—	—					—				
1st extraction of heated suspension	W	+++F	+++	++	++	++	+	—				
	H	—	—	—	—	—	—	—				

* M = mixed agglutination; G = granular agglutination; F = floccular agglutination; W = whole antigen; SH = shaken antigen; H = heated antigen.

After absorption with the deflagellated suspension the flocculating agglutinin titer of "whole" antiserum was decreased by one dilution. In other words the shaken and heated suspensions had removed some flocculating agglutinin in spite of the fact that flagellar antigen was entirely lacking. The third washing fluids gave a slight floccular reaction with the whole antigen in low dilution. Both of the extraction fluids agglutinated the whole suspension with a typical floccular clumping through a dilution of 1:64 (1:1,600). There was no reaction with the heated antigen. The extraction of shaken and heated suspensions of B. paratyphi Type I sensitized by exposure to homologous serum gave practically the same results. The titer of flocculating agglutinin was reduced following absorption and a flocculating agglutinin was removed upon extraction. The extraction tests apparently confirm the earlier observation that deflagellated suspensions of motile bacteria may absorb some flocculating agglutinin from homologous "whole" antiserum.

The fragility of bacterial flagella suggested a possible explanation, other than that of extraction, for the removal of flocculating agglutinin from the sensitized bacteria. The flagella are particularly sensitive to heat, as demonstrated by Beyer and Reagh (6), Orcutt (7) and others. At high temperature the flagella lose their morphological identity and disintegrate. Heated flagellar suspensions while they no longer give a floccular agglutination with antiserum do give a precipitate in low dilutions indicating the presence of soluble material. Although deflagellated motile bacteria may remove some flocculating agglutinin from "whole" antiserum, the bulk of that agglutinin upon absorption by whole bacteria is taken up by the flagella. The removal of flocculating agglutinin from the sensitized bacteria by the method employed might result from a breaking up of the flagella with subsequent release of the previously combined antibody. A number of experiments were performed to demonstrate the possible relationship of flagellum destruction to agglutinin removal.

The effect of temperatures on either side of that employed in the previous extractions and of high salt concentration on the agglutinability of Type II B. paratyphi was first determined.

Heavy suspensions, about twice normal opacity, were prepared in normal saline and in 5 per cent sodium chloride solution. These were heated to 55°,

60° and 65°C. respectively for 1 hour. The heated suspensions were then diluted to normal opacity with saline and tested with "whole" antiserum. The results of the agglutination tests are given in Table IV.

There was no indication that the flagella were affected by the high salt concentration or by a temperature of 55°C. The agglutinability of the organism both in saline and in 5 per cent sodium chloride solution was practically the same as that of a normal unheated sus-

TABLE IV.

Effect of Heat and High Salt Concentration on the Agglutinability of Type II B. paratyphi.

Suspension	Serum dilutions								
	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200
Unheated in saline	CM*	C	++++	++++	+++	+++	++	+	−
Heated 55°C. in saline	CM	C	++++	+++	+++	++	++	+	−
Heated 55°C. in 5 per cent NaCl	CM	C	++++	+++	+++	++	++	+	−
Heated 60°C. in saline	++++M	+++	++	++	+	+	+	±	−
Heated 60°C. in 5 per cent NaCl	++++M	+++	++	++	+	+	+	±	−
Heated 65°C. in saline	++++G	+++	++	+	±	−	−	−	−
Heated 65°C. in 5 per cent NaCl	++++G	+++	++	+	±	−	−	−	−

* M = mixed type of clumping; G = granular type of clumping.

pension. At 60° and 65°C. the destruction of flagella was plainly indicated. At 60°C. both saline and 5 per cent salt suspensions agglutinated to the titer limit of the serum but the intensity of the reaction was much reduced in all dilutions. At 65°C. the type of clumping changed from a predominantly floccular one to a purely granular one. The limiting dilution was much reduced corresponding to that of deflagellated bacteria by "whole" antiserum, as pre-

viously determined. At 65°C. complete destruction of the flagella was indicated.

If the removal of antibody were due to flagellum destruction through the agency of heat then increasing the temperature to 65°C. should result in a greater yield of flocculating agglutinin. On the other hand, reducing the temperature to 55°C. should practically eliminate the removal. A number of extractions were carried out at

TABLE V.

Effect of Varying Temperature and Salt Concentration on the Removal of Agglutinin from Sensitized Type II B. paratyphi.

Method of extraction	Type of antigen	Limit of aggluti-nation	Form of clumping
55°C. in 5 per cent NaCl	W*	1:128	Floccular
	H	<1:2	
55°C. in saline	W	1:64	Floccular
	H	1:2	Granular
60°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
60°C. in saline	W	1:256	Floccular
	H	1:2	
65°C. in 5 per cent NaCl	W	1:526	Floccular
	H	<1:2	
65°C. in saline	W	1:256	Floccular
	H	<1:2	

* W = whole antigen; H = heated antigen.

various temperatures and salt concentrations of the diluent. The agglutinin titer of the several extracts is given in Table V. The results are expressed in terms of the highest dilution showing any macroscopic evidence of agglutination.

Heating the sensitized bacteria to 55°C. resulted in the removal of considerable flocculating agglutinin. As noted in Table IV there was no destruction of flagella at that temperature. The yield of free agglutinin was somewhat greater in the presence of 5 per cent sodium

chloride than in saline. With the latter diluent a small amount of granular agglutinin was removed. At 60°C. the yield of floccular agglutinin was increased with both diluents but there was no further increase at 65°C. The complete destruction of flagella at that temperature was previously noted. From the reported observations the removal of flocculating agglutinin was obviously not dependent solely on the destruction of flagella either through the agency of heat or high salt concentration.

The substitution of distilled water for salt solution in the method of extraction likewise resulted in some liberation of agglutinin. The reaction presented a number of peculiarities concerning the mode of action of the extracted antibody. Experiments bearing on the reaction are being continued.

DISCUSSION.

The preceding experiments indicate that the two agglutinins present in "motile" antiserum display certain differences with respect to their removal from sensitized antigen. It was found that the flocculating or flagellar agglutinin could be freed from its combination with antigen over a considerable range of temperature and salt concentration. Suspension of the sensitized bacteria in a 5 per cent solution of sodium chloride followed by heating to 60°C. for 1 hour was particularly favorable for the removal of flocculating agglutinin. The method of titration was not strictly quantitative but the removal of approximately 50 per cent of the combined agglutinin was indicated. With similar treatment there was little or no removal of granulating agglutinin either from sensitized motile or non-motile bacteria.

It may be said that the combining or clumping properties of the granulating agglutinin of "motile" antiserum are not affected by the conditions imposed upon it during extraction. "Motile" antiserum diluted with 5 per cent sodium chloride solution and heated to 60°C. for 1 hour was found to agglutinate heated homologous antigen in as high dilution as did the untreated serum. The content of granulating agglutinin was not noticeably reduced.

Huntoon and Etris (4) had previously claimed that the presence of salt interfered with the removal of agglutinin from sensitized antigens. Their work was confined to the pneumococcus, the menin-

gococcus and *B. dysenteriæ* Flexner. All of these organisms are non-motile and as antigens give rise to granulating agglutinin but not to flocculating agglutinin. With the foregoing experiments the presence of sodium chloride was found to inhibit the removal of granulating agglutinin from sensitized motile and non-motile bacteria. Increasing the concentration of sodium chloride, on the other hand, increased the amount of flocculating agglutinin removed from the sensitized motile bacteria. A 5 per cent solution of sodium chloride gave a greater yield than did an 0.85 per cent solution. The present findings confirm the work of Huntoon and Etris on the inhibitory effect of salt on the removal of that agglutinin which reacted, in their case with non-motile bacteria, and in the present case with non-motile and deflagellated motile bacteria. Their original statement must be modified, however, to include only granulating agglutinin, since the presence of salt favors the removal of flocculating agglutinin.

It was shown that destruction of flagella through the agency of heat with a subsequent liberation of combined agglutinin could not alone explain the dissociative reaction. It was also shown that flagella were not destroyed by the high concentration of salt. At a temperature of 55°C., which does not affect the flagella, a considerable amount of flocculating agglutinin was removed. At 65°C., which results in complete destruction of the flagella, there was no increase over the amount removed at 60°C. The latter temperature is about the critical point for the beginning destruction of flagella by heat.

The method employed was originally chosen as one favorable for the extraction of globulin. It seems probable that the liberation of flocculating agglutinin attendant upon its application is associated with that protein. Experiments bearing on the chemical nature of the extracted antibody were not attempted. While it was free in the sense that it could again unite with specific antigen it still may have retained some cellular fraction from its previous union. Locke and Hirsch (8) commenting on extraction procedures in general state that in most instances no notable dissociation of the antigen-immune substance union is produced but rather a disintegration and dispersion of the sediment, *i.e.* the sensitized antigen.

Failure to remove granulating agglutinin under the conditions of extraction might be due to differences in the chemical nature of the

two agglutinins or to differences in the manner of linkage to their respective antigens.

SUMMARY.

It was shown that flocculating (flagellar) agglutinin and granulating (somatic) agglutinin display certain differences with respect to their removal from sensitized bacteria (*B. paratyphi*). A 5 per cent solution of NaCl added to sedimented, sensitized bacteria followed by heating to 60°C. for 1 hour removed approximately 50 per cent of the combined agglutinin. There was little or no removal of granulating agglutinin either from the sensitized motile bacteria or from a sensitized non-motile organism (*Staphylococcus*). Evidence was presented that the agglutinin removal was not dependent solely on disintegration of flagella by the conditions of extraction with a subsequent freeing of antibody.

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THE ACID-BASE COMPOSITION OF GASTRIC SECRETIONS.*

By JAMES L. GAMBLE, M.D., AND MONROE A. McIVER, M.D.

WITH THE ASSISTANCE OF PAULINE MARSH AND ELIZABETH MATTESON.

(From the Departments of Pediatrics and Surgery, Harvard Medical School, Boston.)

Excepting as concerns hydrochloric acid, very few measurements of inorganic factors in the composition of gastric secretions have been published. Gamble and Ross (1) found sodium in vomited stomach secretions from a dog following experimental obstruction of the pylorus to the extent of quite one-half the equivalence of the chloride ion present. In a study of the effects of pyloric obstruction in rabbits, Gamble and McIver (2) showed that more than twice the entire plasma content of fixed base was lost into the stomach during the survival period. They regard this extensive withdrawal of fixed base as the cause of the accompanying rapid dehydration of the blood and interstitial body fluids. The data to be here presented were obtained with the purpose of learning whether or not fixed base is contained in stomach secretions, produced under approximately normal conditions, to an extent which would explain a large loss of base in the presence of circumstances preventing reabsorption of these secretions.

So far as we are aware, the only satisfactorily complete analyses of gastric juice are those of Rosemann (3). The material studied was "hunger juice" obtained from dogs by the sham feeding method of Pawlow. An appreciable amount of fixed base was found. The secretion of an alkaline juice by the mucosa of the pyloric antrum was noted by early workers and has recently been confirmed by Ivy and Ayama (4), and by Limb and Dott (5). The production of an alkaline mucous secretion is of course not confined to the pyloric region. Beaumont (6) noted that the inner coat of the stomach of his amiable *voyageur* was "constantly covered with a very thin, transparent, viscid mucus, lining the whole interior of the organ," and that "on applying the tongue to the mucous coat of the stomach, in its empty, unirritated state, no acid taste can be perceived."

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* A partial report of the findings presented in this paper was published in the *Proceedings of the Society for Experimental Biology and Medicine*, 1926, xxiii, 439, under the title, "Fixed base in gastric juice."

The animals used in our experiments were cats. The secretions analyzed were obtained from isolated pouches constructed in the fundic and pyloric regions of the stomach. The animals were operated under ether anesthesia with aseptic technic. During the recovery period, they were cared for in warm, well bedded cages. The pouches were provided with external fistulæ and were found to retain secretions entering them until removed by catheterization. The greater part of the material studied was obtained from an animal in which a pouch of the Heidenhain type had been established in the fundus of the stomach. Samples of juice were taken from this pouch over a period of 2 months, the animal remaining in an excellent state of nutrition and apparently suffering no discomfort.

Chemical Methods Used.

Total fixed base, Fiske (8); *chlorides*, Fiske (9); *inorganic phosphate*, Fiske and Subbarow (10); *potassium and calcium*, Tisdall and Kramer (11). *Sodium* was taken as total fixed base—(potassium plus calcium). The magnesium factor in total fixed base, which according to the measurements of Rosemann (3) is relatively minute, was disregarded. *Nitrogen* was determined by the micro Kjeldahl method of Folin and Denis (12).

The secretions obtained from the pouch during active digestion of food in the stomach were colorless and water-clear. It was found that if the pouch was well drained of these secretions no material could be obtained from it by subsequent catheterization after intervals of fasting. Evidently the secretion of the resting mucosa did not accumulate sufficiently to permit obtaining a sample for analysis. The existence of this secretion could however be made clearly evident by allowing juice produced after ingestion of food to remain in the pouch past the period of active digestion in the stomach. This juice, on being withdrawn, instead of being water-clear always showed a faint but definite white cloudiness, and on analysis a relatively extensive gain in organic material and in fixed base was found. In Table I are given the results of analyses of three large specimens of secretions taken from the pouch at differing intervals after ingestion of food. Each specimen was composed of a number of separate collections of juice. The table also contains Rosemann's data from pooled collections of "hunger juice" from a dog. As may be seen in

the table, the inorganic factors in the juice collected during active digestion of food in the stomach (Specimen 1) correspond closely with the values found by Rosemann for "hunger juice" in the dog. In the specimens composed of juice allowed to remain in the pouch after completion of active gastric function (Specimens 2 and 3) relatively much larger amounts of organic material and of fixed base were measured. This finding together with the change in the physical

TABLE I.

Data from Secretions Taken from Isolated Pouch in Gastric Fundus of Cat and Rosemann's Data for "Hunger Juice" from a Dog.

	Specimen 1 2–4 hrs. after food Water-clear	Specimen 2 8–12 hrs. after food Faint milkiness	Specimen 3 18 hrs. after food Moderate milkiness	Hunger juice from dog Rosemann
	gm. per 100 cc	gm. per 100 cc	gm. per 100 cc.	gm. per 100 cc
Dried substance	0 169	0 282	0 649	0 387
Ash	0 121	0 179	0 384	0 131
Organic substance	0 048	0 103	0 265	0 256
Nitrogen	0 010	0 018	0 041	
Protein (N × 6 25)	0 063	0 113	0 256	
Inorganic substances Cl	0 588	0 574	0 552	0 630
P	0 00016	0 00019	0 00055	0 00027
Na	0 028	0 045	0 128	0 022
K	0 045	0 053	0 053	0 037
Ca	0 0106	0 0057	0 0041	0 00015
Total	0 672	0 678	0 781	0 689
Organic + inorganic substances	0 720	0 781	1 003	0 945

appearance of the specimens from water-clear to "faint" and "moderate" milkiness would seem definitely to indicate admixture of the mucous secretion of the resting mucosa with the acid juice from the fundic glands. The increase of fixed base, it may be noted, is composed of sodium, the values for potassium remaining approximately stationary.

In Table II are given measurements of chloride ion, of fixed base and in a few instances of potassium obtained from single collections of juice from the pouch. The first two groups of measurements are

TABLE II.

Data from Secretions Taken from Fundic Pouch of the Heidenhain Type.

Specimen No.	Food	Interval after food	Volume	Cl′	B·	H·	K·
		hrs.	*cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
1	Meat	1	2	160	22	138	
2	"	2	7	166	17	149	
3	"	3	4	167	24	143	
4	"	6	8	168	44	124	
5	Fish	1	17	177	49	128	
6	"	2	9	156	31	125	12.5
7	"	2	14	169	75	94	
8	"	4	3	157	39	118	
9	"	2	9	171	32	139	
10	"	2	11	169	30	139	11.7
11	"	2	21	173	24	149	11.8
12	"	4	3	152	35	117	11.6
13	Milk	4	28	174	46	128	
14	Bread and milk	2	10	156	41	115	
15	" " "	3	18	176	25	151	
Average......		3.7	11	166	36	140	12
16	Cream	1	3	161	49	112	
17	"	2	2	160	42	118	
18	"	3	2	162	38	124	
19	Cereal and cream	6	10	160	72	88	14.6
20	" " "	6	14	162	77	85	10.4
21	" " "	8	8	157	83	74	9.6
22	" " "	6	7	151	80	70	8.9
23	" " "	2	8	167	71	96	10.9
24	Bread and water	2	4	165	65	100	
25	" " "	4	3	186	55	131	
26	" " "	2	6	162	48	114	
Average......		3.8	6	163	62	101	11
27		18	45	160	53	107	
28	Morning	18	24	155	72	83	12.1
29	specimens	18	13	158	89	69	
30		18	8	157	139	18	15.6
31		18	5	164	142	22	11.8
Average......		18	19	159	99	60	13

from samples of juice taken from the pouch within 6 hours after ingestion of food, with the single exception that Specimen 21, was collected 8 hours after feeding. The food given the animal before collecting the samples in the first group was meat, fish or milk. These were the foods given while collecting the pooled material from which the measurements in Table I were obtained. The second group of data in Table II are from samples of juice produced following ingestion of food low in protein and without appreciable amounts of buffer salts. From the measurements of phosphate and the estimations of protein given in Table I it is evident that these anions are, as compared with chloride, present in negligible amounts. The concentration found for chloride ion minus that for fixed base will therefore closely describe the concentration of hydrogen ions. This datum, taken as Cl'-B·, is also given in the table. The third group of measurements in Table II are from samples of juice taken from the pouch in the morning before feeding, different amounts of food having been given the evening before.

The outstanding finding from these data is a wide range of fixed base concentration in the presence of a fairly stationary concentration for chloride ion. It is thus evident that variation in acidity of the juice as measured by concentration of hydrogen ions is referable to change in fixed base. The data in the first and second sections of the table are from samples taken from the pouch during active digestion of food in the stomach. The considerable difference in the average values for fixed base for the two groups suggests an adjustment of this factor according to the character of the ingested food. The juice produced during digestion of acid-binding foods contains appropriately less of fixed base than is found when foods low in protein and buffer salts are given. It must be noted however that the individual measurements are in both groups often wide of the average value. If the fixed base in the juice is wholly or partly derived from the mucous secretion of the fundus, the collection interval used and the volume of the specimen obtained are probably factors in the extent to which fixed base is added to the juice from the fundic glands. As may be seen in the table, the intervals allowed for secretion of the specimen following ingestion of food were not uniform and also there was an unaccountable variation in the amounts of juice produced. No

regularity of relationship between these factors and the concentrations of fixed base found, can be made out from these data.   By prolonging the collection period past the period of active digestion there occurs, as has already been noted, an increase of fixed base concentration. The data from the "morning specimens" given in the third section of Table II will serve to indicate that the increase of fixed base is fairly closely proportional to the size of the specimen and this finding may be taken as additional evidence that the fixed base found is, in great part at least, derived from the mucous secretion of the fundus.   In Table III are given data from specimens of juice collected from the pouch during the digestion of food to which had been added a relatively

TABLE III.

Data from Secretions taken from Fundic Pouch during Digestion of Food Containing Added Sodium Bicarbonate.

Specimen No.	Food	Interval after food	Volume	Cl'	B·	H·
		hrs.	*cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*	*cc. 0.1 N per 100 cc.*
32		2	4	166	60	106
33	Fish, 30 gm.,	4	8	166	73	93
34	+	6	6	176	50	126
35	NaHCO₃, 2 gm.	2	7	173	53	120
36		4	2	158	43	115
Average.....................		3.6	5	168	56	112

large amount of sodium bicarbonate.   As may be seen, there was no compensating increase in the acidity of juice.[1]   The fixed base concentrations found are actually somewhat higher than when the same food without added alkali was given (first section of Table II).   It is thus evident that we cannot regard the data given in the first and second sections of Table II as dependably indicating that gastric juice acidity is regulated with reference to the character of the food intake by adjustment of fixed base.   The differing amounts of fixed base found are probably due to admixture in varying degree of the

[1] This finding is perhaps of some clinical significance with regard to bicarbonate therapy in gastric hyperacidity in that it may be taken to indicate that sodium bicarbonate is not an aggravating stimulus.

mucous secretion with "true" juice. An accurate control of gastric acidity by a regulated mixing of these two secretions is a conception rather difficult to accept.

The results of an experiment given in Table IV indicate that the production of the mucous secretion of the fundus may be greatly increased by the presence of an injurious agent and suggest that an important rôle of this secretion is protection of the mucosa against

TABLE IV.

Data from Secretions Taken from Fundic Pouch Following Irritation by Alcohol.

Interval after irritation by alcohol	Volume of specimen	Cl'	B·	H·
	cc.	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.
15 min.	2	124	119	5
1 hr.	4	135	99	36
2 hrs.	2	141	90	51

TABLE V.

Data from Secretions Taken from Fundic Pouch Constructed According to the Technic of Pawlow.

Specimen No·	Cl'	B·	H·
	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.	cc. 0 1 N per 100 cc.
37	170	36	134
38	170	30	140
39	175	32	143
40	166	32	134
Average..........	170	32	138

harmful irritation. The experiment consisted in introducing into an empty fundic pouch a small quantity of 95 per cent alcohol which was then almost immediately withdrawn.[2] This procedure induced secretions to an extent sufficient to provide several successive samples large enough for analysis. As may be seen in the table, the chloride

[2] This experiment was carried out with the animal from which the data in Table II were obtained.

ion concentration was greatly below the usual value and was almost covered by fixed base. Over an interval of 2 hours there occurred a considerable increase of chloride and a decrease of fixed base. These data suggest that measurements of fixed base as well as of chloride ion might be informative in the study of gastric anacidity.

In Table V are given data from several specimens taken from a pouch constructed according to the technic of Pawlow, by which vagus innervation of the pouch is preserved. The food given this animal was fish and the specimens were collected 4 hours after ingestion. As may be seen in the table the values found for chloride and fixed base agree fairly closely with those obtained from juice taken from the Heidenhain type of pouch.

In a third animal, a pouch was constructed in the region of the pyloric antrum and from it small amounts of a colorless and viscid secretion were obtained by prolonged catheterization. From a specimen composed of a number of separate collections, the following measurements per 100 cc. were obtained: $B^{\cdot}$, 169 cc. 0.1 N, Cl', 158 cc. 0.1 N, $K^{\cdot}$, 8.8 cc. 0.1 N, dried substance, 2.49 gm. The reaction of the material as determined colorimetrically was pH 8.4. Doubtless, however, this degree of alkalinity was in part due to loss of CO_2. This secretion is thus seen to contain fixed base at approximately the concentration found for chloride ion in juice from the fundus of the stomach, and to owe its alkalinity to a slight recession of chloride ion from this level. The extent to which fixed base is in excess of chloride ion, 11 cc. 0.1 N, may probably be taken as an approximate measurement of bicarbonate ion. In Table II, as in Table I, it may be noted that the values for potassium being approximately stationary, variation in the amounts of fixed base is evidently referable to sodium. The specimen from the pyloric pouch contains potassium at about the level found in the secretions from the fundus. The probability that the mucous secretion of the resting mucosa of the fundus has the same composition as that of the pyloric antrum is thus indicated.

The above data describing the acid-base composition of the gastric secretions are graphically summarized in Fig. 1. In the diagrams, the anions compose the right-hand and the cations the left-hand column. The figure also contains a diagram of the acid-base composition of

cat's blood serum.[3] The first three diagrams illustrate clearly the range of hydrogen ion concentration in secretions from the fundic pouch produced by change in fixed base in the presence of an approximately stationary concentration of chloride ion. It is perhaps interesting to note that whereas in the blood plasma the total ionic content is determined by the fixed base concentration owing to the
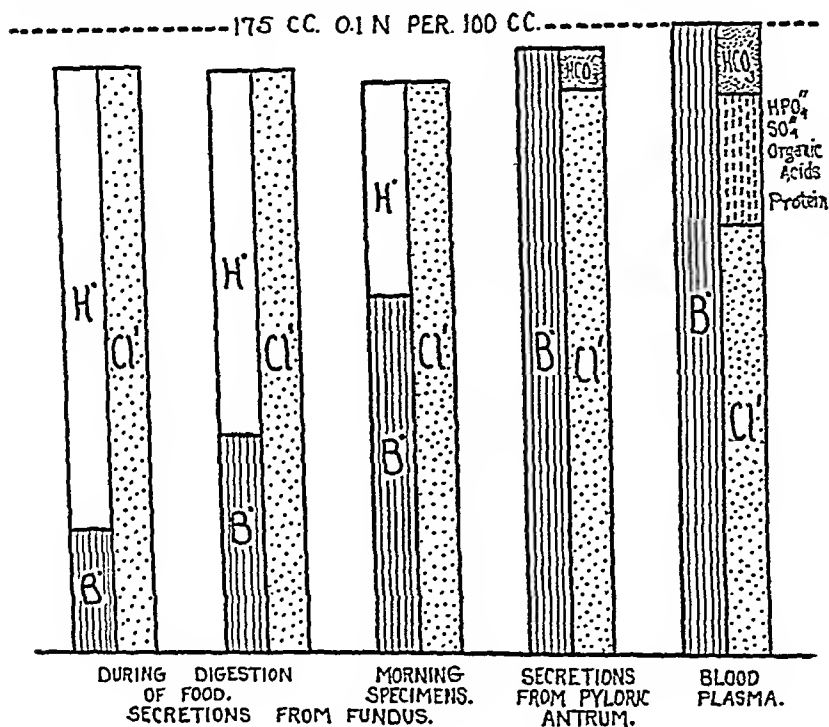
FIG. 1. From data in Table II.

adjustability of the acid factor (HCO_3'), in the secretions of the gastric fundus this value is established by chloride ion and the movable factor sustaining acid-base equivalence ($H^.$) is on the base side of the diagram. An approximate isotonicity of gastric juice and blood

[3] Except for the bicarbonate value which is the average of a few measurements made in this laboratory, the data published by Baumann and Kurland (7) were used in constructing this diagram.

serum has been indicated by measurements of freezing point depression. By comparing the heights of the diagrams in the figure it is evident that according to these data the total ionic content of cat's blood serum is appreciably greater than was found for the gastric secretions. A Donnan relationship between physiological solutions separated by secreting cells would not be expected. The fact, however, that the total of ions is here greater in the solution containing protein and that the chief anion chloride is found at higher concentration in the protein-free solution might be taken as a vague hint of Donnan control of at least the factors defining total ionic content.

A glance at the composition of the gastric secretions as displayed in these diagrams will make easily understandable the large withdrawal of fixed base as well as of chloride ion from the blood plasma which may occur when reabsorption of these materials is prevented. Irritant circumstances in a fasting animal, experimental obstruction of the pylorus for instance, may be expected to produce a relatively large amount of the alkaline mucous secretion. It is therefore not surprising that, as has been found, a chloride loss may be accompanied by more than half its equivalence of fixed base.

SUMMARY.

The chief inorganic factors in secretions obtained from isolated pouches constructed in the fundus and in the pyloric antrum of the cat's stomach were found to be chloride ion and fixed base. In a series of samples obtained from the fundic pouch, chloride ion was approximately stationary at 165 cc. 0.1 N per 100 cc. During digestion of food in the stomach, secretions from the pouch contained fixed base in amounts varying considerably from an average of 47 cc. 0.1 N per 100 cc. Material allowed to remain in the pouch after the completion of food digestion in the stomach showed an increasing content of fixed base, to as much as 140 cc. 0.1 N per 100 cc. A stationary total ionic content of secretions of the fundus is thus seen to be sustained by the chloride ion concentration, and changes in hydrogen ion concentration to be caused by variation of fixed base. The differing amounts of fixed base found are regarded as probably due to admixture of a mucous secretion with the juice from the fundic glands. The alkaline secretion taken from a pyloric pouch contained

fixed base in excess of chloride ion. Variation of fixed base in the secretions from the fundic pouch were found to be referable to change in sodium content, the smaller factor, potassium, remaining approximately constant at about the value found in material from the pyloric pouch. This suggests that the mucous secretion of the fundus has the same composition as that produced by the pyloric antrum. These data will serve to explain the extensive withdrawal of fixed base, as well as of chloride ion, from the blood plasma in the presence of circumstances causing a continued loss of stomach secretions.

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ACID-BASE COMPOSITION OF PANCREATIC JUICE AND BILE.

By JAMES L. GAMBLE, M.D., AND MONROE A. McIVER, M.D.

WITH THE ASSISTANCE OF PAULINE MARSH AND MAURICE M. TOLMAN.

(From the Departments of Pediatrics and Surgery, Harvard Medical School, Boston.)

The measurements of fixed base in gastric secretions given in the preceding paper, explain a rapid depletion of blood plasma base when these secretions are continuously lost from the stomach. It has been pointed out that this loss of fixed base, rather than the larger loss of chloride ion, is the significant factor in the accompanying dehydration of the plasma (1). The alkaline digestive juices entering the duodenum presumably contain more of fixed base than of chloride ion. Under normal circumstances the inorganic substances contained in these secretions are in chief part returned to the blood plasma. If the concentrations of these substances in the digestive juices are appreciable in terms of their blood plasma values, continued failure of reabsorption due to abnormal circumstances such as upper intestinal obstruction, fistula, or diarrheal disease, may be expected to produce dehydration of the plasma and, also, distortion of acid-base structure, unless the amounts of the individual substances lost are proportional to their plasma concentrations. In this study it was undertaken to establish the concentrations of the chief plasma substances, fixed base and chloride ion, in pancreatic juice, in bile from the hepatic duct and from the gall bladder, and in secretions accumulating in an upper intestinal loop. The materials studied were obtained from dogs and from cats by the operative procedures described below. Two specimens of human pancreatic juice were obtained from a patient with a pancreatic fistula.

Collection Methods.

Pancreatic Juice.—An unusual surgical situation provided the opportunity of collecting two samples of human pancreatic juice. This material was obtained

from a patient at the Massachusetts General Hospital, who had undergone an operation for removal of the contents of a large pancreatic cyst. The walls of the cyst were stitched to an opening in the anterior abdominal wall and for a time a sinus tract discharging a clear mucoid fluid persisted. In addition to this material, a clear, watery fluid was noted to leave the sinus after meals. Two small collections of this latter material were obtained following breakfasts of milk, cream, tea, and toast. Tests for the specific ferments of pancreatic juice in the fluid were positive.

A few samples of juice were obtained from the cat, but this animal was found to be unsatisfactory both as regards the establishment of a fistula and the subsequent withdrawal of juice. In the formation of the fistula, it is necessary to use the main pancreatic duct. Since this duct opens into the duodenum at the papilla of Vater close to the bile duct, it is necessary to side-track the bile in order to obtain the pancreatic juice alone. Furthermore, the lumen of the intestine is so small that it is not possible to excise the portion containing the pancreatic duct and then close the wall, so that the intestinal stream, as well as the bile, must be diverted from the first portion of the duodenum. This was done in stages as follows: Under ether anesthesia, an anastomosis was made between the gall bladder and the stomach, and a posterior gastroenterostomy between the stomach and the jejunum was carried out. 4 weeks later, the animal having completely recovered from the operation, the common duct was divided, the bile now passing from the gall bladder into the stomach. The portion of the duodenum containing the pancreatic duct was then excised for transplantation into the abdominal wall as a permanent fistula. The distal end of the duodenum was closed by suture. The stomach contents then entered the intestine through the posterior gastroenterostomy. Except for loss of weight, the animals remained for 2 weeks in fair condition. There appeared to be a continuous slight secretion of pancreatic juice from the fistula which greatly increased on taking food.

The dog with us, as with other investigators, proved in all respects satisfactory. The fistula was established by two methods. The first, that of Pawlow (2), is as follows: A small section of the duodenal wall containing the accessory pancreatic duct is excised; the opening in the duodenum is closed, and the section of the duodenum containing the mouth of the duct transplanted into the abdominal wall.

The second method is an adaptation by Elman and McCaughan (3) of the Rous and McMaster method (4) for collecting sterile bile, and is practically identical with the latter, except that the pancreatic duct is used. Briefly, it consists in cannulation of the pancreatic duct and collection of the secretion in a balloon outside the abdomen. The connection between the cannula and the balloon is made by a somewhat elaborate series of rubber tubes which insures collection of the juice under aseptic conditions. It has the disadvantage, pointed out by Pawlow (2), in regard to methods involving cannulation of the duct, that the cannula acts as a constant irritant, perhaps stimulating abnormal secretion.

Bile.—The bile studied was obtained from cats, collections being made from both the gall bladder and the hepatic duct. The gall bladder collections were made as follows: After a 24 hour fast, the animal was etherized, the abdomen opened and the cystic duct occluded by a clamp. A needle attached to a syringe was then introduced into the bladder and the contents aspirated. The hepatic duct collections were made by an adaptation of the method of Rous and McMaster (4). The gall bladder was first removed and the cystic duct ligated. The hepatic duct was divided and a cannula placed in the proximal end, the end towards the duodenum being ligated. The cannula was connected, by a system of rubber tubing, with a balloon outside the abdomen, an arrangement permitting collection of the entire biliary secretion.

Upper Intestinal Secretions.—A loop about 20 cm. from the pylorus was selected and the bowel divided. A second cut was made through the bowel about 25 cm. distal to the first one, leaving the loop attached by the mesentery only. Great care was used not to interfere with the blood supply. The continuity of the gastrointestinal tract was then restored by an end to end anastomosis and the ends of the isolated loop closed. A small opening into the lumen of the loop was made, and a catheter inserted and held in place by purse string sutures, the free end being brought out through a stab wound in the abdominal wall and connecting with a balloon.

All operations were carried out under ether anesthesia and with aseptic technic. The animals were then cared for in comfortable cages and at the end of the experimental period were killed by etherization.

Methods of Analysis.

The methods used for measuring total fixed base, chloride, inorganic phosphate and calcium, were those cited in the preceding paper. The measurements of sodium and potassium given in Table II were obtained by the method of Stoddard (5) for determining sodium plus potassium and the method of Fiske (6) for potassium.

RESULTS.

The measurements of the two chief factors, fixed base and chloride ion, in the acid-base composition of the secretions studied are given in Table I. The diagrams in Fig. 1 were constructed from average values from these data. The figure also contains for comparison, diagrams representing the acid-base composition of gastric juice and of blood plasma. In the specimens of pancreatic juice, the concentration of fixed base was found to be double or more that of chloride ion. The single measurement of fixed base in human pancreatic juice gave a value only slightly above that usually found for

fixed base in human blood plasma (158 cc. 0.1 N per 100 cc.). The averages for fixed base in the series of samples from Dog 3 and from Dog 4, showed a rather wide difference, being respectively 182 cc. 0.1 N and 159 cc. 0.1 N per 100 cc. Measurements of fixed base in the blood plasma of these animals, however, gave approximately corresponding levels, the values found being for Dog 3, 175 cc. 0.1 N per 100 cc., and for Dog 4, 159 cc. 0.1 N per 100 cc. This degree of difference in the blood plasma level of fixed base in these two animals is surprising and, we believe, unusual. The fixed base found in

TABLE I.

Measurements of Fixed Base and of Chloride Ion.

Material	Number of specimens analyzed	B$^{\cdot}$, cc. 0.1 N per 100 cc.			Cl$'$, cc. 0.1 N per 100 cc.		
		Min.	Max.	Av.	Min.	Max.	Av.
Pancreatic juice, human............	2	—	—	164	86	88	87
" " dog, No. 3.......	19	170	189	182	28	66	42
" " " " 4.......	6	152	163	159	55	89	73
" " cats (two)........	2	192	200	196	67	93	80
Bile, hepatic duct, cats............	11	157	194	172	100	130	120
" gall bladder "	8	261	318	274	0	20	5
Upper jejunal loop, cat*..........	2	169	172	170	129	131	130
Blood plasma, cat**.............		—		175	—	—	119

* Inflammation due to infection was a factor in production of fluid taken from the jejunal loop.

** From measurements published by Baumann and Kurland (7).

single samples from each of the two cats, 192 cc. 0.1 N and 200 cc. 0.1 N per 100 cc., is considerably above the usual plasma concentration in this animal, 175 cc. 0.1 N per 100 cc., established by the data of Baumann and Kurland (7). The specimens of pancreatic juice, however, were small, and the measurements obtained from them cannot be taken as dependably defining the usual concentration of fixed base in pancreatic juice from the cat.

Detail of the acid-base composition of a sample of pancreatic juice from Dog 4 is given in Table II. As may be seen, the fixed base is
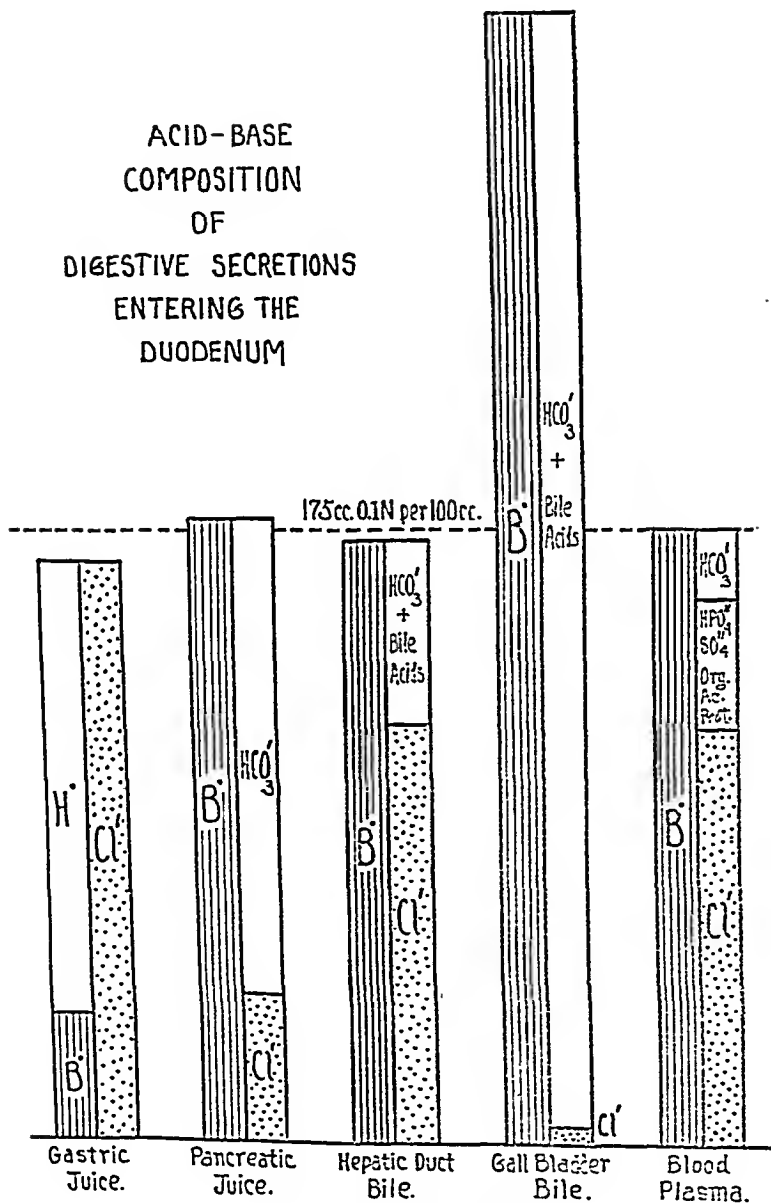
FIG. 1. From data in Table I.

composed almost entirely of sodium, potassium and calcium being
found at relatively small values. Magnesium was not measured.
Except for a minute concentration of phosphate, the acid factors are
chloride ion and bicarbonate ion. In this table the concentration of
HCO_3' is assumed to be measured by the extent to which fixed base
is in excess of $Cl' + HPO_4''$. That this surmise is approximately
correct is indicated by several measurements of bicarbonate obtained

TABLE II.

Acid-Base Composition of Pancreatic Juice from Dog.

HCO_3' taken as total base—$(Cl' + HPO_4'')$. Magnesium not measured.

Base		Acid	
	cc. 0.1 N per 100 cc.		cc. 0.1 N per 100 cc.
Na·	148	Cl'	81
K·	7	HPO_4''	1
Ca··	6	HCO_3'	79
	161		161

TABLE III.

*Measurements of Bicarbonate in Pancreatic Juice after Equilibration with Alveolar
Air Compared with Values for Total Fixed Base Minus Chloride.*

Material from Dog 4.

Specimen No.	B· − Cl'	HCO_3'
	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.
1	96	99
2	88	98
3	81	87
4	63	69

by a somewhat rough method from samples of pancreatic juice in which
fixed base and chloride were also determined. The measurements
were obtained by first shaking samples of juice in the Van Slyke CO_2
apparatus with alveolar air from the operator and then determining
bicarbonate in the usual manner. As may be seen in Table III, the
measurements were somewhat beyond the mark but may be taken as
satisfactorily demonstrating that the acid equivalence of the base in
pancreatic juice is composed practically entirely of chloride and ·
bicarbonate.

The data from the series of specimens of pancreatic juice from Dog 3 and from Dog 4 (Table I) demonstrate that fixed base is, for the individual, a much more stationary value than is chloride ion.   Pawlow (2) and others have stated that the alkalinity of pancreatic juice varies considerably, apparently according to differences in the character of the food intake.   Fixed base remaining stationary, adjustment of bicarbonate and thereby degree of alkalinity, is referable to change in the chloride ion concentration.   The mechanism establishing reaction is thus the inverse of that found in gastric secretions where, as shown in the preceding paper, chloride ion is stationary and fixed base is the movable factor.

In bile from the hepatic duct, fixed base and chloride were found at approximately the concentrations which obtain in the blood plasma (see Fig. 1).   The fixed base concentration in bile from the gall bladder exhibits, interestingly, a wide departure from the blood plasma level, reaching in one of the specimens a value of 318 cc. 0.1 N per 100 cc.   The work of Rous and McMaster (4), which has shown us that the gall bladder has the unique function of concentrating a physiological secretion, would lead us to expect this finding.   Another striking and ingenious change in the composition of gall bladder bile is the almost complete removal of chloride ion, with presumably, replacement by bile acids.[1]

It is the evidence of these data that, during digestive activity in the upper intestinal tract, much more of fixed base than of chloride ion is required.   In the case of pancreatic juice, these materials are directly withdrawn from the blood plasma.   The release of gall bladder bile with its large content of fixed base does not, however, involve an immediate and extensive removal of plasma base.   Replacement of bladder bile is presumably a gradual process extending over the period of absorption of fixed base from the gastrointestinal tract and

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[1] In the diagrams representing the composition of the bile specimens the presence of bicarbonate is indicated.   No measurements of bicarbonate were made.   From the studies of Rous and McMaster of the reaction of bile, we are informed that gall bladder bile is nearly neutral or even slightly acid, and hepatic duct bile, or "liver bile" to use their more appropriate term, is decidedly alkaline.   It may therefore be inferred that the former contains a very small amount and the latter a considerable amount of bicarbonate.

is probably accomplished without disturbing the fixed base content of the plasma. We may thus regard the reservoir mechanism for supplying the digestive secretion of the liver as providing a probably important protection against sudden and large depletions of plasma base. It is to be noted however that, in the presence of circumstances causing continued loss of bile, extensive withdrawal of plasma fixed base must occur unless the food intake provides adequate replacement.

The experiment of constructing a jejunal loop with fistula, continuity of the remainder of the gastrointestinal tract being preserved, was undertaken with the purpose of learning the inorganic composition of the secretions of the upper intestinal mucosa during the digestion of food. The experiment was unsuccessful. It was found that moderate infection and inflammation were undoubtedly factors in the production of the fluid which was obtained from the loop in surprisingly large amounts. The measurements obtained are given in the table for the reason that they probably indicate the composition of the fluid which is so abundantly produced by the intestinal mucosa under obstructive conditions. As may be seen both fixed base and chloride ion are present at approximately their respective concentrations in blood plasma.

SUMMARY.

Pancreatic juice contains fixed base at approximately the concentration found in the blood plasma. Chloride ion is present in concentrations varying from one-fourth to one-half the fixed base value and the remainder of the acid equivalence is composed of bicarbonate ion. Fixed base being a nearly stationary factor, variation of bicarbonate and thereby of alkalinity is referable to change in the concentration of chloride ion.

In bile, as delivered by the liver, both the fixed base and chloride ion values correspond closely with their respective concentrations in blood plasma. In gall bladder bile, however, the concentration of fixed base is, roughly, double that in hepatic duct bile, and chloride ion has been almost entirely removed.

From these data it may be inferred that loss of digestive secretions entering the duodenum will, in the absence of replacement of the

materials contained, cause dehydration of the blood plasma and reduction of the plasma bicarbonate.

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BODY FLUID CHANGES DUE TO CONTINUED LOSS OF THE EXTERNAL SECRETION OF THE PANCREAS.

By JAMES L. GAMBLE, M.D., AND MONROE A. McIVER, M.D.

WITH THE ASSISTANCE OF PAULINE MARSH AND DOROTHY SLOAN.

(From the Departments of Pediatrics and of Surgery, Harvard Medical School, Boston.)

The body fluid changes caused by continued loss of gastric secretions have been fairly well defined by the experimental work of a number of investigators (1–3). The outstanding alterations are dehydration of the interstitial body fluids and of the blood plasma, and alkalosis. These changes are referable to a continued withdrawal of fixed base and of chloride ion from the blood plasma. The data in the preceding paper demonstrate that the digestive secretions entering the duodenum also contain these plasma substances in appreciable amounts. Presumably loss of these secretions, in the absence of adequate replacement of the materials contained, will cause disturbances of body fluid volume and structure. The data to be presented in this paper describe an experimental attempt to define the body fluid changes developing as a result of continued loss of the external secretion of the pancreas.

The general effects accompanying complete loss of pancreatic juice were first observed by Pawlow (4). He found that dogs with pancreatic fistulæ die unless certain measures are taken. To quote from his description of an experiment: "3 to 4 weeks after the operation, the animal previously well to all appearances, became suddenly ill. Food was almost at once refused and a rapidly increasing disability supervened . . . . followed after 2 or 3 days by death. . . . . There remained but one supposition, *viz.*, that the animals, in the escape of pancreatic juice, lost something essential to the normal processes of life." Pawlow made the significant observation that this train of events could be prevented by placing sodium bicarbonate in the food, and also noted that the survival period was much longer on a diet of bread and milk than when meat alone was given. Elman and McCaughan (5) have recently withdrawn and collected the external secretion of the pancreas by an ingenious method involving cannulation of the

duct. The survival period of their animals (dogs) was very much shorter than observed by Pawlow, probably because of the development of persistent vomiting, an event which, according to Pawlow's experiments and our own, does not occur when the external secretion is withdrawn by means of a fistula.

The findings to be here described were obtained from two experiments with dogs. In the first experiment a Pawlow fistula was constructed using the accessory pancreatic duct. The external secretion lost was therefore only a part of the total production. The diet was bread and milk until the 37th day of the experiment when it was changed to meat. Except for a gradual decline in weight, this animal remained in good condition for 38 days. Symptoms of muscular weakness, apathy, and loss of appetite then developed progressively until the animal was *in extremis* on the 42nd day after operation. In the animal used in the second experiment the main and accessory pancreatic ducts were found united before entering the duodenum so that all of the external secretion was drained from an external fistula of the Pawlow type. The diet given was exclusively meat. The survival period was very much shorter than in Experiment I. The animal was active and apparently in good condition for 10 days and then became progressively weaker and more apathetic, and took little or no food or water. 15 days after operation the animal was in serious condition, showing symptoms of narked prostration; extremities cold, pulse rapid, respiration increased in depth and frequency, and blood pressure so low that it was impossible to obtain a blood sample until the femoral artery had been exposed under novocaine anesthesia. At what seemed to be almost the end of the survival period each of these animals was given large amounts of salt solution by subcutaneous injection and intravenous injections of sodium bicarbonate solution. The surgical part of these experiments was carried out under ether anesthesia, and with aseptic technic. The animals were then placed in comfortable cages and distilled water provided for drinking. When the end of the survival period was evidently near, they were killed by etherization.

Chemical Methods Used.

Plasma bicarbonate was determined by the method of Van Slyke (8) and fixed base by the method of Fiske (9). An unpublished method by Fiske was used

in measuring plasma chlorides. Sodium and potassium in urine and in the horse meat used as food were determined together by the electrolytic method of Stoddard (10) and potassium was separately measured by the method of Fiske (11).

The data obtained from these two experiments are presented in Tables I and II and those from Experiment II which describe the acid-base changes found in the blood plasma are repeated graphically in Fig. 1. As may be seen in the tables the earliest change noted is

TABLE I.

Data from Experiment I.

The value R' represents the remainder of the acid equivalence of total fixed base after subtracting $HCO_3' + Cl'$.

Day after operation	Body weight	Food	Blood plasma			
			B^-	HCO_3'	Cl'	R'
	kg.		cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.	cc. 0.1 N per 100 cc.
16	17.6	Bread and milk	175	26	110	39
22	17.8	" " "	175	30	106	39
32	17.2	" " "		24	101	
36	17.2	" " "		24	104	
37	16.8	Meat				
39	16.3	"		21		
41	15.3	"	161	13	105	43
42		"	146		101	
43	15.3	"	139	11	99	29
44*	15.3	Bread and milk				
45**	15.1	" " "	155	21	103	31

* 1000 cc. 0.9 per cent NaCl solution injected into peritoneal cavity. 100 cc. 2 per cent NaHCO₃ solution given intravenously.

** 5 gm. NaHCO₃ given in milk.

decline of body weight. This begins directly following establishment of the fistula and proceeds gradually until the sudden development of anorexia, when it is greatly accelerated. The data on this point are complete only in Experiment II. The plasma factors studied were found not to be appreciably altered during approximately the first two-thirds of the experimental periods. Then, and corresponding with the sudden appearance of the symptoms described above, extensive change in plasma factors developed rapidly.

Except as regards the concentrations of bicarbonate and of chloride ion, these changes resemble closely those produced by continued loss

TABLE II.

Data from Experiment II.

The value R' represents the remainder of the acid equivalence of total fixed base, B', after subtracting $HCO_3' + Cl'$.

Day after operation	Body weight	Meat intake	Water intake	Blood		Blood plasma						
				Hematocrit	Red count	Protein	N.P.N.	Urea	B'	HCO₃'	Cl'	R'
	kg.	*gm.*	*cc.*	*per cent*		*per cent*	*mg. per 100 cc.*	*mg. per 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*	*cc. 0.1 N 100 cc.*
Operation		0				9.4			175	26	105	44
1	20.4	0	460									
2	20.0	624	1440									
3	20.2	567	1120									
4	20.2	454	800									
5	20.0	340	640							25		
6	19.7	1247	1280									
7	19.7	623	1120									
8	19.5	567	960	36	5,300,000				175	22	101	52
9	18.8	737	960									
10	18.7	680	960	41	6,910,000	9.4			161	19	98	44
11	18.2	680	960									
12	17.5	170	0	44	6,570,000	11.6			158	12	96	50
13 a.m.	16.8	170	0	47	6,480,000	12.0			160	12	93	55
p.m.							220	187		12	94	
*14 a.m.	15.8	0	20	44	6,820,000	12.1			159		93	
p.m.				47	7,890,000	11.1	315		161	7	87	67
**15 a.m.	16.1	0	20	47	7,880,000	9.8	290		166	7	103	56
p.m.				43	7,100,000	8.8	325	285	167	17	104	46

* 2000 cc. 0.9 per cent NaCl solution injected into peritoneal cavity.

** 400 cc. 2 per cent NaHCO₃ solution injected into femoral vein.

of stomach secretions. Dehydration of the plasma is indicated by the increase of plasma protein, the rise in red count, and the hematocrit readings (see Table II). Elman and McCaughan (5) in their much
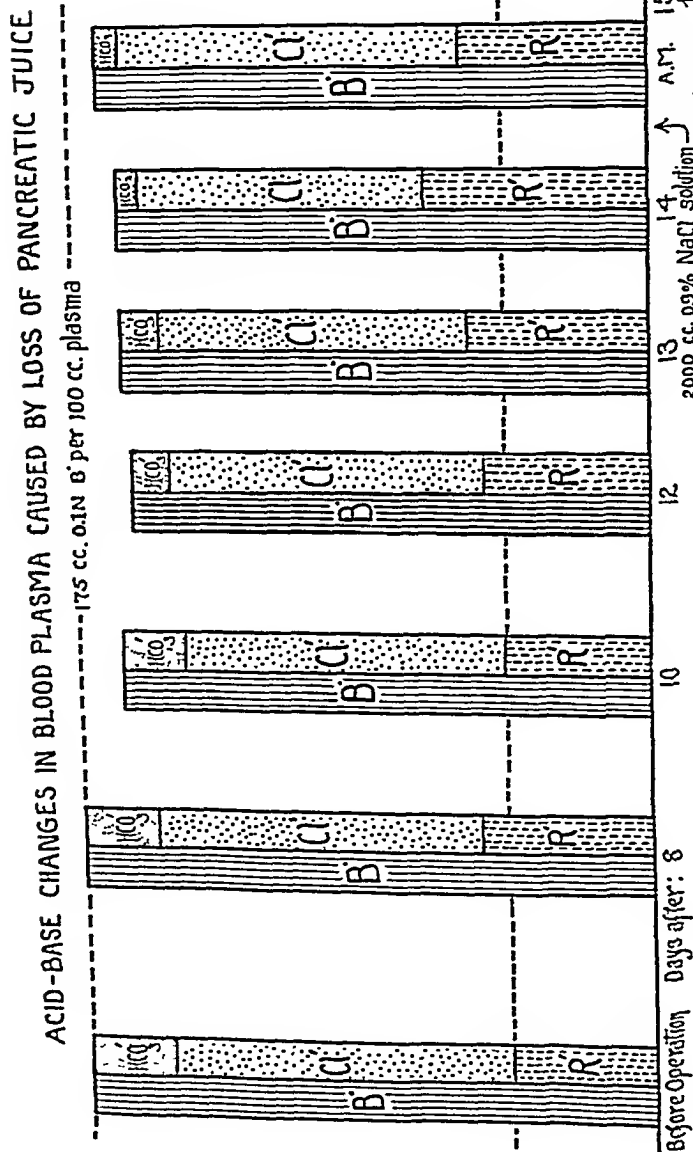
Fig. 1. From data in Table II.

more extensive series of experiments report dehydration of the blood as an outstanding finding. The few measurements of non-protein nitrogen and of urea nitrogen obtained in Experiment II near the end of the survival period show a very large accumulation of protein end-products in the plasma.

A chief purpose of these experiments was to define the changes in the acid-base structure of the plasma caused by loss of a digestive secretion containing much more of fixed base than of chloride ion. Since, when stomach secretions are lost, the relatively much larger withdrawal of chloride ion causes an extension of bicarbonate, it might be expected that loss of pancreatic juice would produce a reduction of bicarbonate.[1] This change was actually found in these two experiments and its relationship to changes in other acid-base factors in the plasma is easily apparent in the diagrams constructed from the measurements obtained in Experiment II (Fig. 1). There occurs a decrease in the concentration of fixed base and an increase in the sum of the acid factors other than the bicarbonate ion and chloride ion (the value R' in the diagrams), just as has been found to occur when stomach secretions are lost. These two changes will cause a reduction of bicarbonate unless accompanied by an equivalent or more than equivalent decrease of chloride ion concentration. This latter event occurs when stomach secretions are lost and explains the bicarbonate increase. When pancreatic juice is lost there is also, according to the data here presented, a decline in chloride ion concentration but not of sufficient extent to prevent a large reduction of bicarbonate. It is of interest to note that, had the chloride ion concentration been sustained at its initial level, bicarbonate would have been entirely obliterated. These points of agreement and of contrast between acid-base changes caused by loss of stomach secretions and by loss of

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[1] The sum of the acid factors in the plasma is kept equal with the sum of the base factors by adjustment of the bicarbonate ion concentration, change in any factor causing change in bicarbonate in the direction which will maintain acid-base equivalence. In the diagrams in Figs. 1 and 2, equivalence is represented by the equal height of the two columns measuring the sum of acid and of base factors respectively, and the extent of change in bicarbonate necessary to maintain equivalence in the presence of change found in other factors is clearly described.

pancreatic juice, may be readily seen by comparing the diagrams in Fig. 2, constructed from data by Gamble and Ross (3) with those in Fig. 1.   These data then demonstrate the expected finding that the continued loss in pancreatic juice of fixed base in excess of chloride ion causes a reduction of plasma bicarbonate.   Elman and McCaughan (5) report that in their experiments a rise in plasma pH was noted.   This would of course indicate a bicarbonate ion concen-

ACID-BASE CHANGES IN BLOOD PLASMA DUE TO LOSS OF GASTRIC SECRETIONS
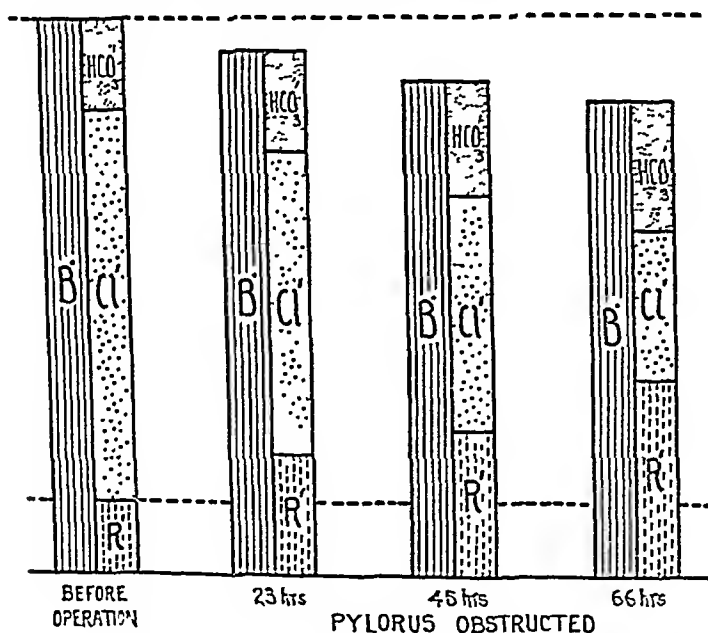
Fɪɢ. 2. From data published by Gamble and Ross (3).

tration higher than usual.   They state, however, that vomiting was a regular and prominent circumstance during the last several days of the survival period, so that unquestionably there was some loss of stomach secretions as well as of pancreatic juice, an event which, as has just been noted, tends to cause extension of the plasma bicarbonate.   Evidently the gist of these findings is that loss of the external secretion of the pancreas produces extensive dehydration

of the plasma and interstitial body fluids and a reduction of plasma bicarbonate in contrast with dehydration and bicarbonate extension caused by loss of stomach secretions.

Dehydration can probably be correctly regarded as the result of a loss of fixed base (3). The fixed base lost in digestive secretions is almost entirely sodium. That a large deficit of fixed base does occur from loss of stomach secretions following pyloric obstruction is easily demonstrable. This may not be directly assumed, however, as a result of withdrawal of pancreatic juice under the experimental circumstances here present for the reason that food was given from which replacement of sodium and of chloride ion can presumably to a certain extent be obtained. Pawlow's observation that ingestion of sodium bicarbonate will greatly, and a milk diet considerably, prolong the survival period obviously suits the conception of a sodium deficit. Meat, the greater part of the fixed base content of which is potassium, which cannot be used to an appreciable extent in the plasma, should be an unsuitable food. The meat given the animals in our experiments was boiled horse meat without added salt. On analysis of several samples it was found to contain per 100 gm.: K, 90 cc. 0.1 N; Na', 11 cc. 0.1 N; and Cl', 17 cc. 0.1 N. From these data and the amounts of meat eaten by the animal in Experiment II during the 11 days preceding the appearance of symptoms and extensive changes in the blood plasma, it may be computed that the average daily intake of sodium was 65 cc. 0.1 N. We have unfortunately no measurements of the daily loss of pancreatic juice from this animal. On this point, however, the extensive data of Elman and McCaughan (5) are available. In a series of twelve experiments they found an average daily production of 250 cc. of pancreatic juice. All of their animals were smaller than the animal used in our Experiment II. This average daily quantity of juice would contain, according to the analyses in the preceding paper, about 370 cc. 0.1 N sodium. If all of the sodium in the ingested meat (65 cc. 0.1 N per day) is assumed to have been absorbed, the estimated daily deficit of sodium from the outset of the experiment is thus approximately 300 cc. 0.1 N. If the loss of this amount of sodium was accompanied by the water which contained it in the interstitial body fluids, dehydration of this animal may be

taken as having proceeded at the rate of about 200 cc. per day.[2] This is admittedly an extremely rough estimation of the probable sodium and corresponding water loss for this animal. It may be noted however that the actual loss of body weight during the first 10 days of the experimental period was approximately 2000 gm. As further evidence of sodium poverty a sample of urine obtained by catheter on the 12th day of this experiment was found to contain relatively minute amounts of sodium and of chloride, as may be seen from the following values per 100 cc. of urine: $K^{\cdot}$, 171 cc. 0.1 N; $Na^{\cdot}$, 1.6 cc. 0.1 N; Cl', 1.3 cc. 0.1 N; PO_4'', 87 cc. 0.1 N.

The experiment of attempting to prevent the appearance of symptoms by early and continued replacement of sodium was not undertaken, our chief purpose being to define the plasma changes. Attempt was however made to repair these changes, at what was apparently almost the end of the survival period, by administration of sodium chloride and sodium bicarbonate solutions. Although in both instances the condition was hopelessly advanced, repair was to a certain extent accomplished and striking though temporary improvement in behavior of the animal was observed. The dog used in Experiment I was, on the 43rd day, extremely sick, showing marked muscular weakness and apathy. Within an hour after administration of 1000 cc. of salt solution by clysis and 100 cc. of 2 per cent sodium bicarbonate solution by intravenous injection, the apathy and muscular weakness disappeared to a remarkable degree. On the following day 5 gm. of sodium bicarbonate were given in milk by stomach tube and a bread and milk diet was substituted for meat. The next morning as may be seen in Table I, there was substantial replacement of plasma base and the bicarbonate concentration was almost doubled. In Experiment II, the animal was almost *in extremis* when the administration of

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[2] The concentration of sodium in the interstitial fluids is for this rough calculation taken as 150 cc. 0.1 N per 100 cc., which is the usual value found in the blood plasma of the dog. The similarity of the inorganic composition of interstitial fluids and blood plasma, except for relatively slight differences referable, in terms of the Donnan law, to unequal concentrations of protein, is indicated by recent studies of the composition of transudates and cerebrospinal fluid (6, 7). For discussion of the limitation of dehydration to interstitial fluids and blood plasma, the reader is referred to papers cited above (3).

repair materials was undertaken. On the afternoon of the 14th day there was marked prostration and respirations were of the type characteristic of severe acidosis. Salt solution, 2000 cc. of 0.9 per cent, warmed to body temperature, was injected into the peritoneal cavity. The animal survived the night and the next morning 400 cc. of 2 per cent sodium bicarbonate solution was injected into the femoral vein. By afternoon there was definite improvement in the physical behavior of the animal. The alterations of plasma factors produced by these solutions are shown in Table II and also by means of diagrams in Fig. 1. It is of interest to note that the sodium chloride solution produced an increase of fixed base and to a somewhat larger extent of chloride ion, but did not alter bicarbonate. Following the injection of bicarbonate solution there was extension of plasma bicarbonate due, curiously, to recession of R', there being only slight further increase of fixed base.

SUMMARY.

From the data given above the following explanation of the effects of continued loss of the external secretion of the pancreas may be offered. The underlying event is a steadily increasing deficit of sodium and of chloride ion due to the large requirement for these electrolytes in the construction of pancreatic juice. In consequence there is continued loss of water, chiefly from the body fluids in which sodium and chloride ion are large factors of total ionic content, viz., interstitial fluids and the blood plasma. During about two-thirds of the survival period the volume and composition of the blood plasma remain approximately normal, the losses of water, sodium, and chloride ion being replaced at the expense of interstitial fluids. Reduction of the volume of these fluids is indicated by loss of body weight beginning directly after establishment of the pancreatic fistula. Ultimately reduction of plasma volume begins and, as it progresses, serious symptoms develop and death occurs unless water, sodium, and chloride ion are abundantly replaced. Owing to the relatively greater loss of sodium than of chloride ion in pancreatic juice, reduction of bicarbonate ion concentration in the plasma tends to occur. The death of the organism may be simply and reasonably explained as the result of progressive impairment of the function of the blood by the physical changes,

dehydration and acidosis, produced in the plasma by the continued loss of sodium and of chloride ion in the pancreatic juice.

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STUDIES IN EXPERIMENTAL EXTRACORPOREAL THROMBOSIS.

VII. Extracorporeal Thrombosis in Experimental Obstructive Jaundice and After the Intravenous Administration of Bile Acids.*

By WALTER R. JOHNSON,† M.D., TAKUJI SHIONOYA‡ M.D., and LEONARD G. ROWNTREE, M.D.

(From the Division of Medicine, Mayo Clinic, and the Mayo Foundation, Rochester, Minnesota.)

PLATE 20.

The prolonged coagulation time of the blood often noted in jaundiced patients has long been recognized as of serious import in relation to surgical measures. Postoperative bleeding, a grave complication, usually occurs when there is delay in coagulation. The coagulation, calcium and prothrombin times are frequently prolonged in obstructive jaundice, particularly if the jaundice has existed for 3 or more weeks. The exact nature of the disturbance in the mechanism of coagulation of the blood, however, is unknown. Available data indicate inhibition of the formation of thrombin and partial inactivation of the thrombin already formed. The calcium and fibrinogen content of the blood, according to most investigators, is undisturbed in these cases.

Purpura in jaundice is also baffling; the coagulation time may be perfectly normal in the face of widespread subcutaneous oozing. Increased capillary permeability, probably toxic in origin, rather than

delayed coagulation seems to be the determining factor. Both hemorrhage and purpura are sometimes terminal events in the course of jaundice. They are also encountered rather frequently in the terminal stages of certain other chronic diseases, notably in nephritis and uremia. Consequently further proof of the specific rôle of retained biliary products in the production of hemorrhage and purpura is legitimately demanded. It is possible that hemorrhage and purpura, like terminal pneumonia, may merely be expressions of impending dissolution. Such a possibility should not be overlooked.

Despite our lack of information concerning the causative mechanism of bleeding in jaundice, certain progress has been made clinically in the control of hemorrhage, postoperative oozing and purpura. Measures aimed at improvement of the patient's general condition are often followed by disappearance of the purpura. An abundance of sugar and water tends, in many cases, to increase the vigor and to decrease the surgical risk. The repeated administration of calcium chloride intravenously sometimes seems to offer protection against subsequent hemorrhage. Such measures combined tend to shorten the coagulation time of the blood and render the patient a more favorable subject for surgical intervention. If these procedures fail, there yet remains transfusion of blood, one of the most satisfactory general methods in the treatment of hemorrhage. But in a considerable number of cases, all efforts at control prove futile.

The experimental procedures were carried out in two series: (1) extracorporeal thrombosis in experimental jaundice, (a) old technic, eight experiments, (b) modified technic, ten experiments, and (2) extracorporeal thrombosis following the intravenous injection of bile acids, (a) single injections of bile acids, (b) continuous injection of bile acids with Woodyatt pump.

Extracorporeal Thrombosis in Experimental Jaundice.

The common duct of rabbits was ligated under ether anesthesia under strictly aseptic conditions. 2 to 5 days later the animals were anesthetized with tertiary trichlorobutyl alcohol, the extracorporeal loop was attached and observations made on the rate and character of formation of the thrombus. Jaundice was apparent in all of the animals on the day of experiment.

Old Technic, Eight Experiments.—The four glass sections of the extracorporeal loop were coated with paraffin but the cannulas and rubber collars were left with clean ungreased surfaces. In the normal animal circulation through the extracorporeal loop ceases in from 6 to 10 minutes, or at most, in unusually large and vigorous animals, in 25 minutes and the collodion tube is completely obstructed by a solid thrombus. If the jaundice has existed for several days the course is quite different. White thrombi composed of platelets and, later, leucocytes are laid down in about the usual manner and can be seen grossly in from 10 to 15 minutes. The formation of fibrin, however, is greatly delayed and the lumen does not become occluded for several hours. White thrombi multiply in size and numbers, chiefly on the venous side, and in some experiments pile up into cauliflower-like excrescences (Fig. 1) in the collodion tube and venous cannula so that the speed of the current is materially decreased and fibrin threads are permitted to become attached firmly. The fibrin in its turn enmeshes erythrocytes, especially in the dependent portions of the apparatus and a thin film of poorly organized thrombus results. The white thrombi in the uppermost portions of the collodion tube retain their identity much longer before being overlaid by fibrin and erythrocytes. Circulation persists in most instances for from 3 to 4 hours.

Modified Technic, Ten Experiments.—The apparatus was first assembled and then dipped in melted paraffin. Only the cannulas and the cannula collars remained unparaffined. In the normal animal with this type of preparation, circulation persists for 2 or 3 hours. White thrombi grow in the cannulas and collodion tube and after an hour or two are found overlaid by red thrombi which ultimately obstruct the lumen.

In the jaundiced rabbit the course is similar except that the flow persists for a much longer period and the white thrombi often retain their identity strikingly, without admixture of red elements or gross fibrin. Even after 3 hours of circulation the membrane may exhibit nothing but a heavy deposit of platelets and leucocytes. The thrombi tend to be laid down in a characteristic fashion being strictly localized at either end of the collodion tube so that they comprise a ring form (Fig. 2). In some experiments, the primary ring-like group of white

elements was later covered by a heavy mass of erythrocytes and fibrin which ceased growing as soon as the disparity in lumen between the glass and collodion tubes was overcome. The inner surface of the mixed thrombus was composed of a smooth fibrin sheath, practically colorless because of the washing action of the circulating blood. The reason for the peculiar localization of thrombus elements at either end of the collodion tube is obscure. It has not been observed in any experimental condition other than obstructive jaundice; probably mechanical and physical factors are responsible.

Bilirubin estimations and bile acid determinations by the quantitative Pettenkofer method of Aldrich and Bledsoe were made before the experiments were begun (Table I). During the experiment the serum bilirubin averaged 7.5 mg. and the bile acids 18.5 mg. for each 100 cc. of blood. The coagulation time was uniformly prolonged.

Type Protocol (Modified Technic).—The common duct was ligated August 27, 1927, and the experiment performed 4 days later when the serum bilirubin was 9.4 mg. and the level of the bile acids 17.5 mg. for each 100 cc. of blood. At 9.50 a.m. the clotting time (Lee and White method) was 9½ minutes. The extracorporeal loop with unparaffined cannulas was attached. The blood flow through the loop was brisk and the formation of eddies was marked. At the end of 10 minutes a few tiny white thrombi could be seen at the venous end of the collodion tube. At the end of 20 minutes these had grown definitely in size and numbers and a few could also be seen in the arterial end. At the end of an hour, the flow was still quite brisk. At either end of the collodion tube were many tiny white thrombi; they could not be seen in the middle portion of the tube. After 2 hours of circulation through the extracorporeal loop, the thrombi had increased in numbers and size so as to form a ring about 1 cm. wide at each end of the collodion tube. After 3 hours the ring at the venous end was distinct and white and that at the arterial end had been overlaid by red thrombi. The blood flow remained brisk and strong and apparently there was no obstruction at any point in the apparatus. The clotting time was 10 minutes.

The apparatus was detached and washed gently with a solution of sodium chloride. The collodion tube was slit longitudinally and flattened. The massed white thrombi at the venous end were creamy white, at the arterial end the red thrombus was covered by a smooth fibrin lining which was washed white by the action of the flowing blood (Figs. 3 and 4). The middle portion of the tube was free of thrombi.

Comment.—During the progress of the experiments certain facts became apparent. It was found, for example, that the condition of

the animal exerted a profound influence on the rate and character of formation of the thrombus in the loop. If the animal was vigorous and healthy and the circulation correspondingly brisk, the flow persisted for a long time and white thrombi only were deposited. On the other hand, if the animal was exhausted from long continued jaundice or was depressed by overdosage of an anesthetic, with consequent weak circulation at the beginning of the experiment, the white thrombi were soon overlaid by fibrin and erythrocytes and the lumen became occluded relatively early. Changes in the quality of the blood and variations in coagulation time appeared to exert a lesser influence than did the purely mechanical factors. For these reasons it was deemed unwise to attempt to draw too far reaching conclusions concerning specific factors. Furthermore, the course of events did not always follow the typical course anticipated. Thus in two experiments performed on animals in whom the common duct had been ligated 3 and 6 days respectively before the experiment, the results were wholly at variance with those usually encountered. In both experiments, on repeated attachment of the apparatus, the lumen became occluded in some portion within 20 minutes and large masses of loose, friable, coiled fibrin were found long before numerous white thrombi had appeared. These animals were in excellent condition and seemed to fulfill all the requirements for experimentation. In a third experiment, circulation through the loop persisted for 10 hours without occlusion. It was obviously impossible adequately to correlate such divergent results. To disregard them would be altogether unwise. That experimental obstructive jaundice and the blood changes accompanying it exert a definite influence on coagulation and the formation of thrombi in the extracorporeal loop seems proved beyond doubt, but at the same time physical and mechanical factors assume such large proportions that they approach, in importance, the purely intrahemic, chemical and physiologic abnormalities induced by the presence of jaundice. Hence in clinical jaundice the general condition of the patient as well as the results of laboratory tests of the blood would seem of great significance.

Extracorporeal Thrombosis Following the Intravenous Injection of Bile Salts.

It has been postulated at various times that the increased level of bile acids of the blood may be responsible for the prolonged coagulation time sometimes noted in cases of jaundice. Petrén, Morawitz and Bierich have maintained that such an hypothesis is untenable since bile salts added to blood *in vitro* have no effect on the coagulation time until the concentration is several times that in obstructive jaundice. Clinical studies carried on in our wards have failed to show any direct parallelism between the level of the bile acids as indicated by the Pettenkofer reaction and the coagulation time of the blood. It was thought that results of interest might be obtained by the use of the extracorporeal loop in animals that had received a solution of bile salts intravenously. Single and continuous injections of pure bile salts dissolved in physiologic sodium chloride solution were made.

Single Injection of Bile Salts.—An adequate single injection of bile salts (5 to 10 cc. of a 1 per cent solution of the sodium salts of the bile acids in physiologic sodium chloride solution) caused a definite prolongation of the coagulation time of the blood of a rabbit. As might be expected, the circulation through the loop persisted longer than normal. White thrombi were laid down in a fairly normal manner, although somewhat delayed, and after an hour or two were found overlaid by erythrocytes and small amounts of fibrin. The circulation, however, continued and there was less tendency to obstruction than occurs in the normal animal.

The rise in the level of bile acids of the blood is transitory after a single injection of pure bile salts, as has been shown by Greene and Snell in their experiments on dogs. If, as has been suggested, the duration of increase in the level of bile acids of the blood is of greater significance than the actual level attained, it would be of interest to keep animals at a constant high level of bile acids for several days and determine whether or not this factor alone could alter the coagulation time as does obstructive jaundice. A prolonged experiment of this type was not attempted, but by means of the Woodyatt pump, it was possible to approximate and maintain, for several hours, the levels of the bile acids found in clinical obstructive jaundice.

Continuous Injection of Bile Salts with Woodyatt Pump.—Five experiments were performed.

A 2.5 per cent solution of bile salts was administered in three experiments, 2 per cent in one and 6.6 per cent in the other, and specimens of blood were removed at intervals to determine the level of the bile acids (Pettenkofer value). The coagulation time was also studied and the extracorporeal loop was attached after a definite clotting disturbance was manifest. The coagulation time was not taken until about 200 mg. for each kilo of body weight had been given. At this point the coagulation time was always found to be increased, although the level of bile acids of the blood, as determined by the quantitative Pettenkofer method of Aldrich, averaged only a few milligrams higher than the normal level of 3 to 4 mg. for each 100 cc. of blood. The great quantity of cholates introduced into the circulation (200 to 300 mg. for each kilo each hour) caused hemolysis in only one experiment, in which a 6.6 per cent solution of bile salts was used. The bile acid content of the blood rose slowly the first 2 hours and reached its highest level at the close of the experiment when the animal was moribund.

The condition produced approximates in some respects that observed in jaundice. The results of the individual experiments are shown in Table II and Text-fig. 1. In Table III a general summary is tabulated. Under the conditions of these experiments from 600 to 700 mg. of bile acids for each kilo were given over a period of about 4 hours. The average coagulation time was prolonged from 3 to 15 minutes and the level of the bile acids increased from the normal of 3 to 4 mg. to an average of approximately 60 mg. at the end of the experiment. The animals became weaker and appeared to be comatose as the experiments drew to a close. Necropsy did not reveal a trace of generalized purpura or hemorrhage.[1]

In the extracorporeal loop white thrombi were never as prominent as in the normal or jaundiced animal. A few small patches of platelets could sometimes be seen grossly but as the blood pressure became

[1] In later experiments it was found that when 4 per cent solution of bile salts was injected into the marginal vein of a rabbit's ear there was a subsequent extravasation of hemoglobin or erythrocytes into the tissues surrounding the smaller venous channels. Grossly the condition seemed to be a purpuric manifestation. The vein used for the injection became thrombosed. The same results were obtained by injecting the central artery of the ear. Sellards has shown that a 5 per cent solution of bile salts injected intraperitoneally causes hemorrhagic areas and necrotic ulcers in the stomach. He could not reproduce these results by intravenous injection since the animals died immediately if a lethal dose was used. Repeated sublethal intravenous injections were precluded by venous thrombosis.

TABLE I.

Changes Observed following Ligation of the Common Duct (Series b).

Experiment No.	Days after obstruction	Average coagulation (Lee and White)	Serum bilirubin	Bile acids (Pettenkofer value)
		min.	*mg. per cent*	*mg. per cent*
Normal		3 to 4	0.0	4.0
1	5	13	7.5	13.2
2	3	13	8.7	13.4
3	4	18	6.6	10.9
4	3	18	8.1	39.0
5	5	10	3.7	16.6
6	4	10	9.4	17.5

TABLE II.

Changes Observed following Continuous Injection of Bile Salt Solution.

Experiment No.	Time	Bile salts injected	Bile acid in blood	Coagulation (Lee and White)
	hrs.	*mg.*	*per cent*	*min.*
8			3.8	3.0
	1.5	600	25.6	6.0
	2.5	1000	20.0	6.0
	3.5	1250	50.0	13.0
	4.5		27.0	15.0
9			3.9	3.0
	0.45	800	15.0	4.0
	2.00	1350	25.0	9.0
10				2.5
	1.25	270	4.9	9.0
	3.25	620	13.9	12.0
	4.25	780	22.2	15.0
	5.00	872	68.5*	15.0
11			4.1	2.5
	1.20	858	13.8	3.5
	2.20	1452	32.2	9.0
	3.20		30.0	15.0
	4.00		80.0*	

* Postmortem specimens.

progressively lower, these patches were overlaid with erythrocytes and thus when the membrane was removed, nothing but red thrombi could be seen. In one experiment this secondary sedimentation of erythrocytes did not obtain and the membrane remained practically clear except for a faint haze of tiny white thrombi and ungrouped platelets.

Type Proctocol (Continuous Injection of Bile Salts).—(Experiment 7). The animal used in the experiment was normal; its weight was 2600 gm. The coagulation time before the experiment was 4 minutes (Lee and White). The content of bile acids in the blood was 3.6 mg. for each 100 cc. The injection of 2.5 per cent bile salts was begun at 2.45 p.m.; at 3.50 p.m. 250 mg. had been introduced.

TABLE III.

Results of Experiments with Continuous Injection of Bile Salts.

Experi-ment No	Solution injected	Bile salt injected	Weight of animal	Bile salts	Duration of experiment	Coagulation		Bile acids	
						Before experiment	After experiment*	Before experiment	At end of experiment
	per cent	*mg.*	*gm*	*mg. per kg.*	*hrs.*	*min.*	*min.*	*mg per cent*	*mg. per cent*
7	2.5	1675	2600	640	5.5	4.5	21	3.6	65.6
8	2.5	1250	1900	658	4.5	3.0	15	3.8	50.0
9	2.5	1350	2900	466	2.0	3.0	9	3.9	25.0
10	2.0	1872	1300	670	5.0	2.5	15		68.5
11	6 6	1452	2750	537	3.5	2.5	15	4.1	80.0

* The greatest delay noted, that is at the end of the experiment usually.

The coagulation time was 6 minutes. The extracorporeal loop was attached at 3.50 p.m. At 4.20 p.m. there was an obstructing clot in the venous cannula. The apparatus was detached and cleaned. White thrombi could not be seen in the collodion tube. At 5.30 p.m. 1250 mg. had been introduced. The coagulation time was 12 minutes. Slight hemolysis was apparent. A new extracorporeal loop was attached at 5.30 p.m. At 6.00 p.m. the level of bile acids in the blood was 21 mg.; 1650 mg. had been introduced. The coagulation time was 10 minutes. White thrombi could not be seen in the apparatus. At 7.00 p.m. the level of the bile acids was 31 mg. The animal was comatose. The coagulation time was 20 minutes; 2650 mg. of bile salts had been given. At 8.30 p.m. the level of the bile acids was 65.6 mg.; 3675 mg. of bile salts had been administered. The animal was moribund. The coagulation time was 21 minutes. A few white thrombi were seen in the collodion tube. The membrane was almost clear.

Neither general hemorrhage nor purpura could be found at postmortem examination.

Text-fig. 1 illustrates the relationship between the quantity of bile salts introduced, the level of bile acids in the blood and the coagulation time in this experiment. The data from the other four experiments presented in Table II form similar curves when plotted graphically.
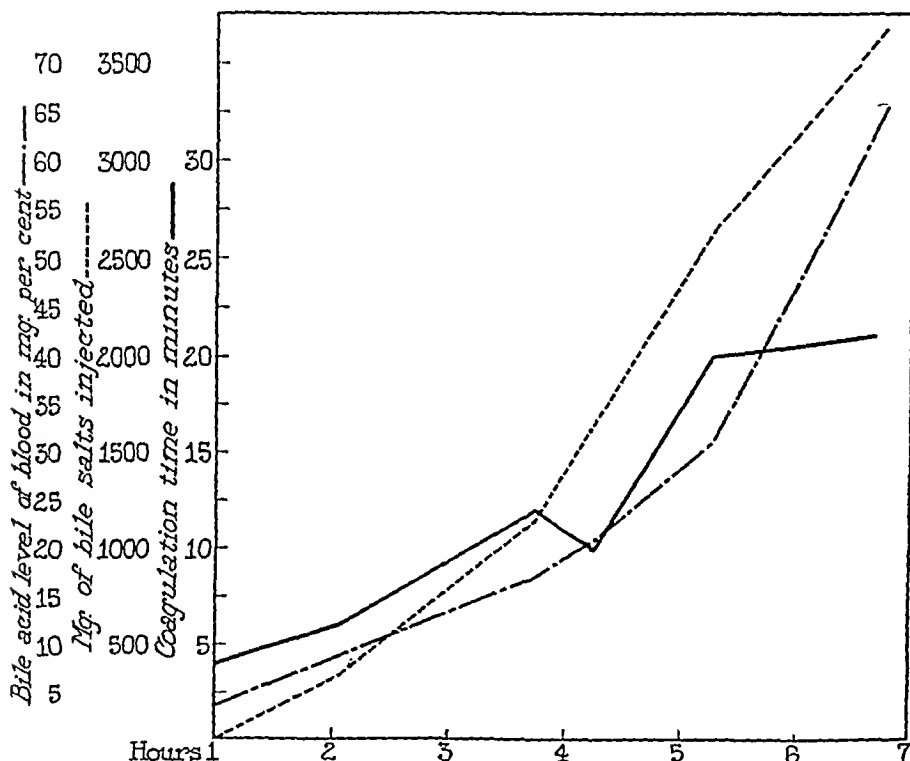
TEXT-FIG. 1. Relationship between the quantity of bile salts introduced, the level of bile acids in the blood and the coagulation time.

Comment.—It is interesting to speculate on the disposal of the huge excess of bile salts introduced into the circulatory system. Obviously they do not remain in the blood, for in this experiment only 21 mg. for each 100 cc. of blood were recovered after 1650 mg. had been injected. It may be assumed that the hepatic cells remove the excess bile acids rapidly and efficiently at first and then, as the injection continues, with less ease until finally death ensues. Greene and Snell in their experiments on dogs found that after a rapid single injection

of a large quantity of bile salts, there was a sudden and profound rise in the level of bile acids of the blood. This rise was short-lived and the level returned to normal within an hour or two.

DISCUSSION.

In the experiments with the Woodyatt pump the Pettenkofer value of the blood rose slowly. The injection was slow and continuous. Despite the relatively low level of bile acids at the end of the 1st hour, the coagulation time of the blood was uniformly prolonged, not markedly, yet in most instances definitely (Table II). As the injection continued, this delay in coagulability usually became progressively more marked. This raises the question of whether bile salts *in vivo* have an effect on the coagulation time of the blood which they do not exert *in vitro*.

Morawitz and Bierich, in 1906, showed that when solutions of the sodium salts of bile acids were added to samples of blood *in vitro* it was necessary to obtain a concentration of 0.5 per cent bile acids to increase the coagulation time to twice the normal value. A concentration of 0.25 of 1 per cent (250 mg. in 100 cc. of blood) did not have any appreciable effect on coagulation time. They concluded that the salts of bile acids act exactly as do neutral salts, that is, the concentration is the factor of importance and there is no specific effect. Petrén, in 1920, performed similar experiments using, however, oxalated plasma instead of whole blood. He stated that a concentration of 0.17 to 0.22 per cent was the borderline value and that 0.55 to 0.66 per cent of bile acids had a definite inhibitory effect on the coagulation of oxalated plasma. He concluded that the increased levels of bile acids found in patients with jaundice could not be responsible for "cholemic bleeding" since coagulation *in vitro* did not appear to be affected until the concentration of bile acids was several times greater than that found in jaundice. In this connection our results from the continuous injection of bile salts are of some interest. Table II and Text-fig. 1 show that the Pettenkofer value of the blood is only a little higher after the gradual injection of 200 to 400 mg. of bile salts (Experiments 7 and 10) than it was at the beginning of the experiments, yet the coagulation time is increased. In Experiments 8, 9 and 11 a much larger quantity of salts was injected during the first

hour and the Pettenkofer value is correspondingly higher; but even then the values are negligible in comparison with those of Morawitz and Bierich and Petrén. As the level of the bile acids of the blood rises during the experiments, the coagulation time is correspondingly lengthened. The highest level of the bile acids obtained (80 mg. per cent in a postmortem specimen) is well below the 0.25 per cent and 0.17 to 0.22 per cent stated by Morawitz and Bierich, and Petrén, respectively, to have a minimal effect on the coagulation of blood *in vitro*. This fact would point to the conclusion that there is some factor other than the physical concentration of bile acids which influences the clotting time in animals that have been given injections of bile salts. Petrén thought that cholemic bleeding was due to a disturbance of the function of the liver. It would seem possible that the absorption of large quantities of bile salts (such as were administered during these experiments) by the tissues, particularly the liver, might so disturb the intimate chemical and physiologic balances of the blood as to effect a delay in coagulation time. No attempt is made to dispose of the question by such an explanation but the results of these experiments would indicate that an indirect rôle of bile acids in the production of delayed coagulation time and cholemic bleeding has not yet been disproved.

An obvious criticism of our experimental methods is the large total quantity of fluid which was injected. In most instances it averaged half the calculated blood volume of the animal. This was balanced somewhat by the periodic withdrawal of blood for determinations of coagulation time and of bile acids. The effect of hemolysis which was observed definitely in one case must also be considered.

<div align="center">SUMMARY.</div>

In experimental obstructive jaundice in rabbits, thrombosis is definitely delayed in the extracorporeal loop. White thrombi are laid down at the normal rate in many instances; in others there is a delay in deposition of platelets, while in still others cauliflower-like masses of platelets are rapidly deposited and tend to slow or even stop the blood stream before much fibrin is apparent. Fibrin deposits and formation of the red thrombus usually appear late in the experiment and often only at the ends of the collodion tubes. In vigorous animals,

the thrombi rarely tend to obstruct and usually cease growing when they have made up the difference between the lumen of the glass tube and of the collodion tube. On the other hand, if the animal's general condition is poor and its circulation depressed, red thrombus forms more rapidly and tends to encroach progressively on the lumen until obstruction takes place. Even in such cases circulation through the loop persists for periods much longer than in the normal animal. Judging from the gross appearance of the thrombus the quantity and quality of fibrin seem deficient.

Single injections of bile salts yield pictures similar to those seen in experimental obstructive jaundice. White thrombi are deposited but obstruction of the lumen is delayed because of inadequate formation of fibrin. The continuous intravenous injection of bile salts resulted in a progressive increase of the clotting time and in the level of bile acids of the blood. The state of the extracorporeal loop was likewise similar in many respects to that seen in obstructive jaundice. There was, however, a definite decrease in the size and numbers of white thrombi deposited on the collodion membrane.

From the evidence presented it is obvious that the processes of blood coagulation and of thrombosis in the extracorporeal loop are definitely delayed in experimental obstructive jaundice and in animals that have received intravenous injections of bile salts. No attempt is made to explain the changes found in jaundice on the basis of the increased levels of bile acids in the blood although these experiments would indicate that such a possibility has not been ruled out.

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EXPLANATION OF PLATE 20.

FIG. 1. Vertical section through a thrombus mass found on collodion membrane. *a*, platelet collections; *b*, thin layer of leucocytes on the surface of collodion membrane (which was dissolved in the fixing process); *c*, red cells.

FIG. 2. Collodion tube slit longitudinally and flattened out. Heavy deposit of platelets and leucocytes at one end of tube; the remainder of tube clean and transparent.

FIG. 3. Collodion tube slit longitudinally and flattened out. Deposit of thrombi at either end of tube. · *a*, washed fibrin lining of red thrombus (torn away when tube was flattened out); *b*, red thrombus; *c*, white thrombus.

FIG. 4. Cross-section of thrombus found at arterial end of tube (Fig. 3). *a*, smooth layer composed of fibrin and leucocytes; *b*, network of fibrin threads with entangled red cells and a few leucocytes; *c*, collodion membrane.
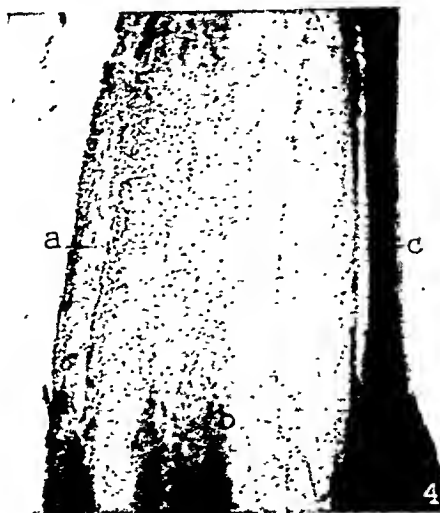
THE PRODUCTION OF EXPERIMENTAL TYPHOID FEVER IN THE GUINEA PIG WITH AN IN VIVO PREPARED TOXIC FILTRATE OF B. TYPHOSUS.*

By WILLIAM H. HARRIS, M.D., AND OGILVIE M. LARIMORE, M.D.

(From the Department of Pathology of Tulane University, New Orleans.)

PLATES 21 TO 23.

The experiments of Grunbaum (1) and of Metchnikoff and Besredka (2) have demonstrated that when *Bacillus typhosus* is introduced into anthropoid apes, certain evidences of human typhoid fever can be reproduced. Positive blood cultures, agglutination reactions, fever, lesions of Peyer's patches and other features of the disease have been reproduced in this type of animal. The experiments carried out with this organism upon the smaller and more practical laboratory animals have, however, as a whole, proven · unsatisfactory. Although a septicemia, peritonitis and death can be readily produced in such smaller animals, the resultant picture bears little if any relationship to the human disease.

Besredka (3) has found that smaller animals primarily prepared by feeding bile are vulnerable to the ingestion of typhoid bacilli and present a more protracted infection with certain features allied to human typhoid. More recently Sedan and Herrmann (4) have employed the injection of typhoid bacilli into the subconjunctival tissues. They have produced by this means a continued fever, diarrhea, tumefaction of Peyer's patches and certain other features analogous to human typhoid although the microscopic study does not appear identical with that found in the human infection. Gory and Dalsace (5) have also reported typhoid infection in the guinea pig, employing the method of Sedan and Herrmann. Although they state that the human disease has been duplicated, a review of their pathological study shows that multiple abscesses were produced in the liver, spleen and the lymphoid tissues of the peritoneal cavity including

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Peyer's patches. They also specify that acute cholecystitis was almost constant and acute pericarditis was at times present. Their results indicate that through the method employed they prolonged the duration of the usual septicemia, permitting thereby the formation of widespread acute exudative lesions. Such lesions do not conform with the host response characteristic of the human disease, the histopathology of which was first most fully described by Mallory (6).

In addition to numerous transmission experiments performed with the organism proper, extensive experimentation has been carried out with the toxic products. Evidence has been advanced in support both of its endotoxic and ectotoxic nature. A great variety of methods have been employed for the purpose of obtaining a specific toxin. A general review of the literature appertaining to the toxin of the organism is to be found in the work of Gay (7).

Persistent difficulty has been encountered in procuring a true specific toxin for many microorganisms, especially those regarded as endotoxic in nature. In this connection, Duval and Hibbard (8) have reported the effects produced in animals by the injection of a toxic material obtained from the streptococcus of scarlatina by means of an *in vivo* process. In their method the supply animal was first immunized by several injections of the streptococcus administered at weekly intervals. It occurred to us that the employment of the animal host in some intermediary capacity may be essential in the production of a toxic material more closely allied to that demonstrated in the typhoid toxemia of man.

When the typhoid bacillus is introduced into the peritoneal cavity of normal guinea pigs an exudative peritonitis is produced. In this conflict of the invading microorganism and the animal host the usual resultant features of an acute inflammatory reaction are found. It appeared likely that in this field of activity in which many factors both of host and invader have been put into action, a toxic substance might be produced which when injected into animals would yield a different character of response than that obtained by the living microorganism.

The experiments herein reported demonstrate the results obtained by the injection into guinea pigs of the toxic factor procured through this *in vivo* method.

EXPERIMENTAL.

A peritonitis was produced in guinea pigs by the inoculation of cultures of
B. typhosus. The virulence of the strain was exalted by passage through several
generations of guinea pigs so that within 7 to 10 hours after injection the animals
became very sick and the abdomen distended with exudate. The animals were
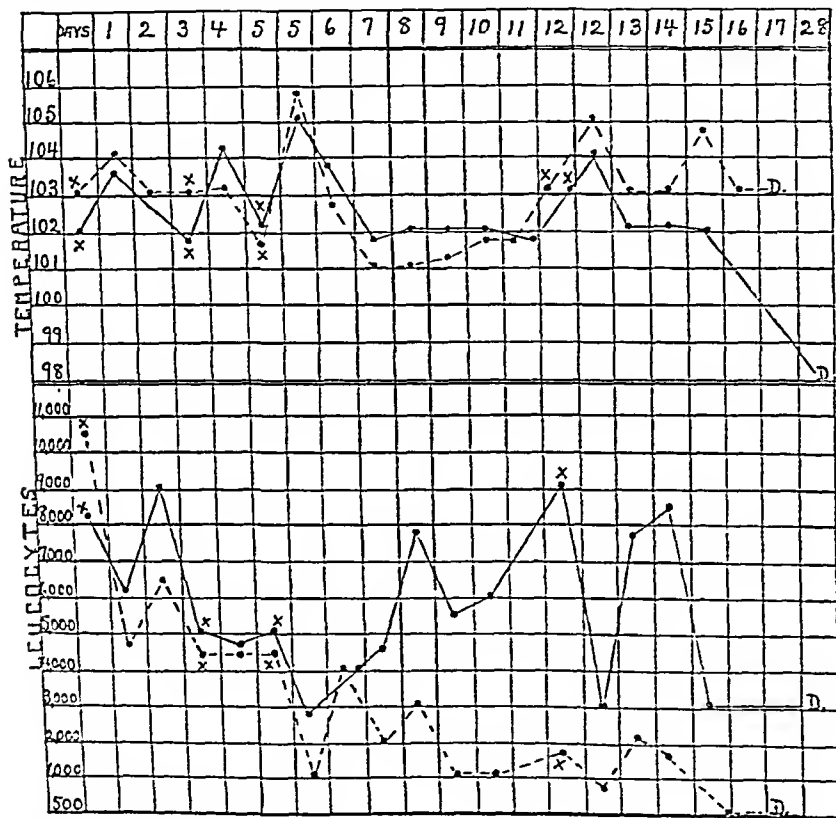
x = Toxin Injections

CHART 1.

then sacrificed and the fluid was withdrawn from the peritoneal cavity. The
exudate varied from a slightly smoky yellow liquid to one of a cloudy, flaky char-
acter, rich in cellular elements. This material was diluted to approximately
four times its volume with sterile water and filtered through an N Berkefeld filter.
The resultant bacteria-free filtrate was employed for animal injection. It was
found preferable to employ freshly prepared filtrate at each interval injection.

Twenty-four guinea pigs have thus far been employed. The protocols of six representative animals of this group have been selected. Of these six, two received the injections subcutaneously, two intraperitoneally and two intracardially. All six animals died in approximately 2 to 4 weeks. The autopsy findings and microscopic study are described under pathology.

Experiment 1.—Two guinea pigs weighing approximately 250 gm. were given four subcutaneous injections of *in vivo* prepared toxin as follows: Each received 4 cc. as a primary injection, 3 cc. 3 days later, 5 cc. on the 5th day and 4 cc. on

X = TOXIN INJECTIONS
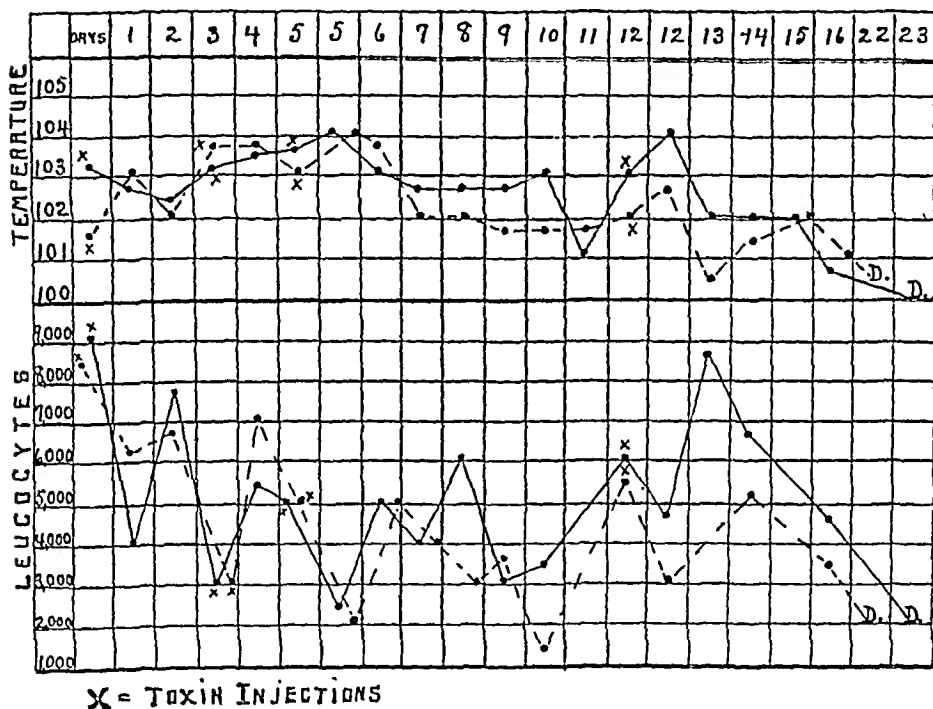
CHART 2.

the 12th day. One of these animals died on the 17th day and the other 11 days later. The temperature and leucocytic counts of each are shown in Chart 1.

As can be seen, subsequent to the injections there usually occurred a rise in temperature and a drop in the leucocytic count. One of the animals showed a drop of the count to 500 cells and the other to 3000 cells per c. mm.

Both animals gradually lost in weight and their appetite became poor. No other clinical manifestations were observed.

Experiment 2.—Two guinea pigs were given four intraperitoneal injections of *in vivo* prepared toxin on the same days as those of Experiment 1 but the following respective amounts were administered, 2 cc., 2 cc., 3 cc. and 4 cc.

The febrile and leucocytic reactions in these two animals were quite consistent and closely parallel. The leucocytic count in both instances dropped to nearly 2000 cells per c. mm. with frequent counts of around 3000 as can be seen in Chart 2. These animals lost in weight, one showing considerable emaciation. One animal died on the 22nd and the other on the 23rd day following the inoculations. No diarrhea was observed in either animal.
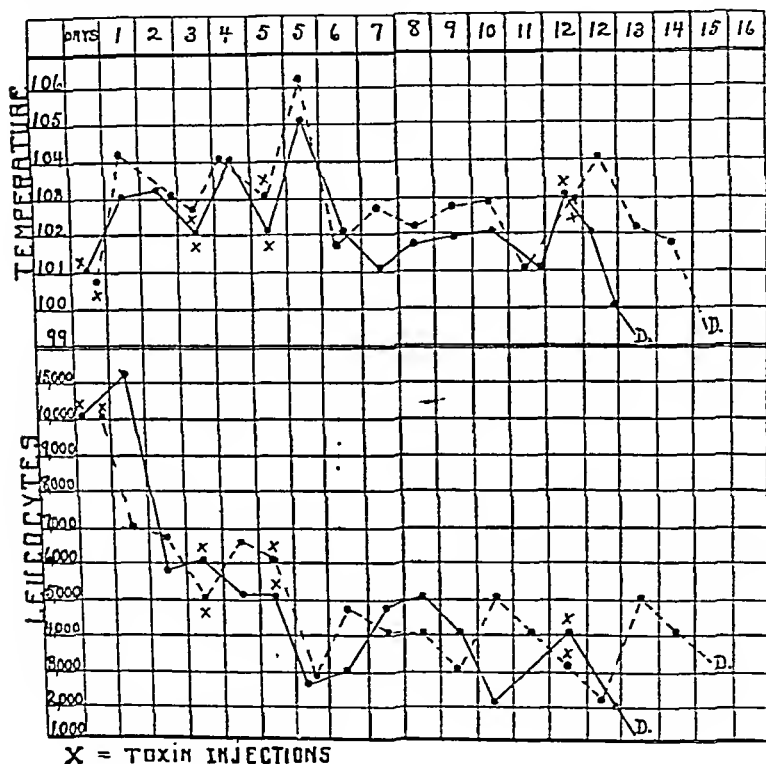
CHART 3.

Experiment 3.—Two guinea pigs were inoculated intracardially with the *in vivo* prepared typhoid toxin. They each received on the 1st day, 1 cc. of the toxin, on the 3rd day, 1 cc. of the toxin, on the 5th day, 2 cc., and on the 12th day, 1.5 cc. Their febrile response and leucocytic decline were marked and followed constantly the injections. The fever rose as high as 106.4°F. for one animal and 105°F. for the other. The leucocytes dropped below 3000 per c. mm. on several occasions, the original counts before injection being approximately 10,000 cells per c. mm. One of these animals died on the 13th day and the other

on the 15th day. The reactions by this route of inoculation were sharper (see Chart 3) and the animals died more quickly.

It can be seen from a review of the charts of these six animals that as a whole the clinical records are analogous in character. With each injection of the toxic material, irrespective of the route of administration, a rise of temperature and a drop of the leucocytes occurred. The febrile response was of a transient character lasting for 2 or 3 days after each inoculation. The abatement of the fever is attributable most likely to the fact that no continued toxemia or accumulative toxic action existed since no living virus was injected. In the instance of the leucocytes, however, after the first injection of toxin the total count seldom returned to the normal; the leucopenia continued and became accentuated subsequent to each inoculation. It would appear that the particular factors or mechanism essential for the production of leucopenia was continued over a longer period of time than the phenomena producing the fever. In no instance was diarrhea observed and aside from loss of appetite and weight and progressive weakness together with the leucocytic and febrile response no other clinical features were discernible.

Pathology.

The postmortem examination of the animals in which death followed the typhoid toxin inoculations showed as a whole the same general gross picture. In certain of the animals the changes were more accentuated than in others but this variation was only in degree or extent and not in the character of the lesions.

At the inoculation site of the animals injected subcutaneously there was found a congested and edematous area about 2 to 3 cm. in diameter. Small hemorrhagic extravasations were at times noted.

The abdominal cavity showed the lymphatic glands to be greatly enlarged and in some of the glands marked congestion and hemorrhage were present. (See Fig. 1.)

Spleen.—This structure was usually of a deep red color and moderately increased in size. The consistence was soft and the pulp stripped off readily, although two of the six spleens were quite firm. No evidences of miliary abscess formation or areas of necrosis were found.

Intestines.—The examination of the intestinal tract showed throughout a

marked enlargement of all lymphoid structures. The solitary follicles protruded well into the lumen and were frequently congested. The Peyer's patches were greatly elevated (see Figs. 2 and 3), often reddened and at times showed slight ulceration. Occasionally a marked necrosis and ulceration of the patch occurred. The fecal content in these areas was fluid in character but no blood was observed.

Liver.—This organ was enlarged, congested and friable and of a deep red color. Scattered throughout the structure were found yellow focal areas varying in size from 1 to 3 mm. The gall bladder showed nothing of note.

Kidneys and Adrenals.—These structures were somewhat swollen and the vessels showed considerable congestion. Evidences of yellowish discolorations suggestive of degeneration were sometimes found.

Bone Marrow.—This was more deeply reddened than normal marrow of the guinea pig.

Heart and Lungs.—No gross lesions were observed. In no instance was pneumonia found present.

Nothing else of note was observed in the gross study of other structures of the body.

Microscopic.—The voluntary muscle in the region of the subcutaneous inoculation showed hyaline or waxy necrosis of this structure. Scattered wandering monocytes or "endothelial" cells were seen about the necrotic muscle. The enlargement of the lymphatic glands especially of the peritoneal cavity and of the solitary follicles and Peyer's patches was found to be due to the hyperplasia of the lymphoid structures and to the accumulation of "endothelial" cells. Many "phagocytic cells" of Mallory were noted in which the engulfed elements consisted of portions of lymphoid cells, nuclear fragments and at times erythrocytes. In certain of the lymphoid structures marked congestion, hemorrhagic extravasations and areas of necrosis were seen. Some of the Peyer's patches showed a loss of the mucosa and necrosis of the cellular elements of the enlarged nodule with destruction of the muscularis mucosæ. (See Figs. 4 to 6.) Sections of the spleen demonstrated marked congestion of the pulp and in some of the animals, hemorrhages were found in this structure. Escape of the hemoglobin was noted in masses of the erythrocytes presenting thereby shadow or phantom corpuscles. (See Fig. 7.) Scattered throughout the splenic structure but particularly in the pulp were endothelial cells many of which showed phagocytosis especially of the shadow red cells.

The liver sections revealed degenerative changes including areas of focal necrosis. These necrotic areas showed no special zonal location occurring centrally or in the portal areas or extending through both zones, and they varied in their size and extent. In some of these areas hemorrhage was present and many of the extravasated cells were located within the protoplasm of the phagocytic cells. The smaller areas of focal necrosis showed replacement of the hepatic cells by the endothelial type of cell. (See Fig. 8.)

The kidneys showed congestion of the vessels and degenerative changes in the

epithelium of the uriniferous tubules. The bone marrow showed marked congestion and at times hemorrhagic extravasations. Nothing noteworthy was found in other structures examined. It is of interest to note that no polymorphonuclear neutrophils or other elements of an acute exudative inflammation were found in the various lesions studied.

Controls.—In a previous article (9) we reported parallel experiments in which *B. coli communior* was employed. Fifteen guinea pigs were used and the results obtained therein were unlike those obtained with *B. typhosus.* Evidences of toxemia with degenerative and hemorrhagic lesions were found. Although death was produced by the toxin of this microorganism, the gross and microscopic aspect did not resemble the human typhoid lesion.

In this connection, the paratyphoid epizootics occurring in the guinea pig should be mentioned. The lesions of this infection as observed by one of us (10) and as studied by Howell and Schultz (11) do not resemble the lesions herein described. Work upon the effects in guinea pigs of the toxic material prepared in a similar manner from paratyphoid bacilli is under way.

<div align="center">DISCUSSION.</div>

In general microorganisms belonging to the so called endotoxic group do not yield *in vitro* a satisfactory specific toxin. This has greatly interfered with the progress of the study of the true nature and effects of such microorganisms. For example, the preparation of a specific toxin from the streptococcus considered as the cause of scarlet fever has been attended with difficulties. Dochez (12) considers that this microorganism contains at least two varieties of poisons. Duval (8), on the other hand, employing his *in vivo* method regards the toxin procured by him from the streptococcus of scarlatina as the true specific toxin of this microorganism. In the instance of the typhoid bacillus, the same general difficulty of obtaining a specific poison has been encountered although numerous and diverse methods have been applied.

A definite toxemia is manifestly present in typhoid fever and the toxin produces in the human host a specific pathological picture. It is not improbable that such a microorganism forms its specific

toxin only when invading its natural host, in other words during its function of pathogenesis. A different or more complete biological process may be evolved *in vivo* as contrasted with *in vitro* activity. Again the somatic cells of the invaded host may play some essential rôle in the production of the specific pathogenic toxin. Because of these possibilities of a differential nature of such toxins, the *in vivo* method was employed. It is believed that the toxic material obtained through this process closely simulates in its action on the inoculated host the activities of the typhoid toxin as evidenced in the human disease.

SUMMARY.

When the typhoid bacillus is injected into the peritoneal cavity of guinea pigs acute peritonitis and death are produced. The character of the exudate is variable as to the elements present but is usually of a serous type with slight clouding due to the presence of polymorphonuclear neutrophils, mononuclear cells and bacteria. When the Berkefeld filtrate of this exudative material is inoculated into normal guinea pigs either subcutaneously, intraperitoneally or intracardially, the character of response obtained on the part of the host is quite at variance with that produced by the inoculation of the living typhoid bacillus. A febrile reaction and marked leucopenia, as a rule, are persistent and are accentuated after each injection the latter often reaching below 1000 cells per c. mm. There is a loss of weight of a variable extent in all animals and in some the emaciation is extreme. The animals were given four such inoculations and all succumbed in from 2 to 4 weeks. The intracardiac route produces death more quickly and the reactions are more clear-cut when this route is employed. At autopsy a general tumefaction and congestion of the lymphoid structures more especially of the abdominal cavity are found. Peyer's patches and the solitary follicles of the intestinal tract are likewise involved and in some of the patches slight ulceration is noted; occasionally, there occur extreme ulceration and necrosis of the patch. The spleen is enlarged and usually softened. Microscopically, marked endothelial cell proliferation is noted especially in the lymphoid structures and in many instances the phagocytic cells of Mallory are found. These cells include within their

cytoplasm elements of the surrounding structures. In the spleen there are present congestion, and hemorrhages with many "shadow" red blood cells. Phagocytosis of the red cells by the endothelial cells is present. In the liver, areas of focal necrosis are found and phagocytic cells are seen. In the animals inoculated subcutaneously, localized degenerative changes are observed especially in the muscular structures.

From these results it can be seen that the reactions and injury of the animal body by the toxic filtrate employed, are quite similar to the changes produced by the specific toxin in human typhoid fever.

CONCLUSION.

During the activity of peritonitis produced in the guinea pig by means of *Bacillus typhosus*, there is formed in the exudative material a filtrable toxic moiety which when inoculated into normal animals of this species, produces certain of the clinical phenomena and a pathological picture simulating that of human typhoid fever.

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EXPLANATION OF PLATES

PLATE 21.

FIG. 1. Lesions of mesenteric lymph glands. These structures are greatly increased in size; congestion and hemorrhage are at times present.

Fig. 2. Specimen of a portion of the ileum showing Peyer's patch with enlargement and elevation of the structure and early ulceration at the upper portion. (Enlarged for detail.)

Fig. 3. Lesion similar in character to that in Fig. 2, with somewhat more marked swelling.

Fig. 4. Lesion of Peyer's patch showing marked swelling of the patch, and in certain areas, there is a loss of the epithelial lining. (Low power.)

PLATE 22.

Fig. 5. Cellular elements of swollen Peyer's patch. Cells of the phagocytic type of Mallory, which contain lymphoid cells and nuclear fragments, can be seen. (High power.)

Fig. 6. High power of lesion of the lymphatic gland, demonstrating the character of the proliferating cells.

PLATE 23.

Fig. 7. Section of spleen showing congestion and hemorrhage. The erythrocytes for the most part are of the "shadow cell" type (loss of hemoglobin) and some are engulfed by phagocytes.

Fig. 8. Area of focal necrosis in the liver. The hepatic cells have been replaced by the endothelial cells which have phagocytized fragments of red blood cells and nuclear portions. (High power.)

(Harris and Larimore: Experimental typhoid fever.)

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